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# *COVER SHEET FOR PROVISIONAL APPLICATION FOR PATENT*

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Commissioner for Patents
P.O. Box 1450
Mail Stop Provisional Patent Application
Alexandria, VA 22313-1450

Sir:

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(c).

| CAM 301891-999221 | Docket Number | 9301-229-888 | Type a plus sign (+) inside this box 6 | + |
|---|---|---|---|---|

| INVENTOR(s) APPLICANT(s) | | | |
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TITLE OF THE INVENTION (280 characters max)

METHOD OF COLLECTING PLACENTAL STEM CELLS

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ENCLOSED APPLICATION PARTS (*check all that apply*)

| | | | |
|---|---|---|---|
| ☒ Specification | *Number of Pages* | 239 (including 153 pages sequence listing) | ☐ Applicant claims small entity status, see 37 CFR §1.27 |
| ☒ Drawing(s) | *Number of Sheets* | 10 | ☐ Other (specify) |

METHOD OF PAYMENT (*check one*)

| | |
|---|---|
| ☐ A check or money order is enclosed to cover the Provisional filing fees. | ESTIMATED PROVISIONAL FILING FEE AMOUNT ☒ $160 ☐ $80 |
| ☒ The Commissioner is hereby authorized to charge the required filing fee to Deposit Account Number 503013. | |

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.
☒ No. ☐ Yes, the name of the U.S. Government agency and the Government contract number are:

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☐ Additional inventors are being named on separately numbered sheets attached hereto.

Total number of cover sheet pages.

## *PROVISIONAL APPLICATION FILING ONLY*

CAJD: 501212.1

**Express Mail No.: ER 505 058 315 US**

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Application of: DAI et al.
Application No.: To be assigned
Filing Date: On Even Date Herewith
For: CLASSIFICATION OF BREAST CANCER PATIENTS USING A COMBINATION OF CLINICAL CRITERIA AND INFORMATIVE GENE SETS

Confirmation No.: To be assigned
Art Unit: To be assigned
Examiner: To Be Assigned
Attorney Docket No.: 9301-229-888

**TRANSMITTAL OF SEQUENCE LISTING**

MAIL STOP PATENT APPLICATION
Commissioner For Patents
P. O. Box 1450
Alexandria, VA 22313-1450

SIR:

In connection with the above-identified application, and in accordance with 37 C.F.R. § 1.821, Applicants submit herewith a Sequence Listing in paper and computer-readable format pursuant to 37 C.F.R. § 1.821(c) and (e).

I hereby state that the content of the paper and computer-readable copies of the Sequence Listing, submitted in accordance with 37 C.F.R. § 1.821(c) and (e), respectively, are the same.

Date March 5, 2004

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Enclosures

Express Mail No.: ER 505 058 315 US
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# CLASSIFICATION OF BREAST CANCER PATIENTS USING A COMBINATION OF CLINICAL CRITERIA AND INFORMATIVE GENE SETS

## 1. FIELD OF THE INVENTION

[0001] The present invention relates to the use of both phenotypic and genotypic aspects of a condition, such as a disease, in order to identify discrete subsets of patients for which specific sets of informative genes are then identified. The invention also relates to the classification of individuals, such as breast cancer patients, into a subset of the condition on the basis of clinical parameters and the status of markers, for example, of genes expression patterns, and the prognosis of those individuals on the basis of markers informative for prognosis within the subset of the condition. The invention also relates to methods of determining a course of treatment or therapy to an individual having, or suspected of having, a condition, such as breast cancer. The invention further relates to methods of structuring a clinical trial, particularly using five breast cancer-specific patient subsets and prognosis-informative genes for each, and of identifying patient populations for clinical trials or for other condition-related, for example, breast cancer-related, research. Finally, the invention relates to computer implementations of the above methods.

## 2. BACKGROUND OF THE INVENTION

[0002] The increased number of cancer cases reported in the United States, and, indeed, around the world, is a major concern. Currently there are only a handful of treatments available for specific types of cancer, and these provide no guarantee of success. In order to be most effective, these treatments require not only an early detection of the malignancy, but a reliable assessment of the severity of the malignancy.

[0003] The incidence of breast cancer, a leading cause of death in women, has been gradually increasing in the United States over the last thirty years. Its cumulative risk is relatively high; 1 in 8 women are expected to develop some type of breast cancer by age 85 in the United States. In fact, breast cancer is the most common cancer in women and the second most common cause of cancer death in the United States. In 1997, it was estimated that 181,000 new cases were reported in the U.S., and that 44,000 people would die of breast cancer (Parker *et al., CA Cancer J. Clin.* 47:5-27 (1997); Chu *et al., J. Nat. Cancer Inst.* 88:1571-1579 (1996)). While mechanism of tumorigenesis for most breast carcinomas is largely

unknown, there are genetic factors that can predispose some women to developing breast cancer (Miki *et al., Science*, 266:66-71(1994)).

[0004] Sporadic tumors, those not currently associated with a known germline mutation, constitute the majority of breast cancers. It is also likely that other, non-genetic factors also have a significant effect on the etiology of the disease. Regardless of the cancer's origin, breast cancer morbidity and mortality increases significantly if it is not detected early in its progression. Thus, considerable effort has focused on the early detection of cellular transformation and tumor formation in breast tissue.

[0005] A marker-based approach to tumor identification and characterization promises improved diagnostic and prognostic reliability. Typically, the diagnosis of breast cancer requires histopathological proof of the presence of the tumor. In addition to diagnosis, histopathological examinations also provide information about prognosis and selection of treatment regimens. Prognosis may also be established based upon clinical parameters such as tumor size, tumor grade, the age of the patient, and lymph node metastasis.

[0006] Diagnosis and/or prognosis may be determined to varying degrees of effectiveness by direct examination of the outside of the breast, or through mammography or other X-ray imaging methods (Jatoi, *Am. J. Surg.* 177:518-524 (1999)). The latter approach is not without considerable cost, however. Every time a mammogram is taken, the patient incurs a small risk of having a breast tumor induced by the ionizing properties of the radiation used during the test. In addition, the process is expensive and the subjective interpretations of a technician can lead to imprecision. For example, one study showed major clinical disagreements for about one-third of a set of mammograms that were interpreted individually by a surveyed group of radiologists. Moreover, many women find that undergoing a mammogram is a painful experience. Accordingly, the National Cancer Institute has not recommended mammograms for women under fifty years of age, since this group is not as likely to develop breast cancers as are older women. It is compelling to note, however, that while only about 22% of breast cancers occur in women under fifty, data suggests that breast cancer is more aggressive in pre-menopausal women.

[0007] In clinical practice, accurate diagnosis of various subtypes of breast cancer is important because treatment options, prognosis, and the likelihood of therapeutic response all vary broadly depending on the diagnosis. Accurate prognosis, or determination of distant metastasis-free survival could allow the oncologist to tailor the administration of adjuvant chemotherapy, with women having poorer prognoses being given the most aggressive treatment. Furthermore, accurate prediction of poor prognosis would greatly impact clinical

trials for new breast cancer therapies, because potential study patients could then be stratified according to prognosis. Trials could then be limited to patients having poor prognosis, in turn making it easier to discern if an experimental therapy is efficacious.

[0008] To date, no set of satisfactory predictors for prognosis based on the clinical information alone has been identified. Many have observed that the ER status has a dominant signature in the breast tumor gene expression profiling. *See* West *et al., Proc. Natl. Acad. Sci. U.S.A.* 98:11462 (2001); van 't Veer *et al., Nature* 415:530 (2002); Sorlie *et al., Proc. Natl. Acad. Sci. U.S.A.* 100:8418 (2003); Perou *et al., Nature* 406:747 (2000); Gruvberger *et al., Cancer Res.* 61:5979 (2001); Sotiriou *et al., Proc. Natl. Acad. Sci. U.S.A.* 100:10393 (2003). It is generally accepted that there is some relationship between patient survival and ER status. van de Vijver *et al., N. Engl. J. Med.* 347:1999 (2002); Surowiak *et al, Folia Histochem. Cytobiol.* 39:143 (2001); Pichon *et al., Br. J. Cancer* 73:1545 (1996); Collett *et al., J. Clin. Pathol.* 49:920 (1996). *BRCA1* mutations are related to the familial cancer susceptibility. Biesecker *et al., JAMA* 269:1970 (1993); Easton *et al., Cancer Surv.* 18:95 (1993). Age is also considered to be a prognosis factor since young cancer patients tend to have poor tumors. Maggard *et al., J. Surg. Res.* 113:109 (2003). Lymph node status is a factor in deciding the treatment. Eifel *et al., J. Natl. Cancer Inst.* 93:979 (2001).

[0009] The discovery and characterization of *BRCA1* and *BRCA2* has recently expanded our knowledge of genetic factors which can contribute to familial breast cancer. Germ-line mutations within these two loci are associated with a 50 to 85% lifetime risk of breast and/or ovarian cancer (Casey, *Curr. Opin. Oncol.* 9:88-93 (1997); Marcus *et al., Cancer* 77:697-709 (1996)). Only about 5% to 10% of breast cancers, however, are associated with breast cancer susceptibility genes, *BRCA1* and *BRCA2*. The cumulative lifetime risk of breast cancer for women who carry the mutant *BRCA1* is predicted to be approximately 92%, while the cumulative lifetime risk for the non-carrier majority is estimated to be approximately 10%. *BRCA1* is a tumor suppressor gene that is involved in DNA repair and cell cycle control, which are both important for the maintenance of genomic stability. More than 90% of all mutations reported so far result in a premature truncation of the protein product with abnormal or abolished function. The histology of breast cancer in *BRCA1* mutation carriers differs from that in sporadic cases, but mutation analysis is the only way to find the carrier. Like *BRCA1*, *BRCA2* is involved in the development of breast cancer, and like *BRCA1* plays a role in DNA repair. However, unlike *BRCA1*, it is not involved in ovarian cancer.

[0010] Other genes have been linked to breast cancer, for example c-erb-2 (*HER2*) and p53 (Beenken *et al., Ann. Surg.* 233(5):630-638 (2001). Overexpression of c-erb-2 (*HER2*) and

p53 have been correlated with poor prognosis (Rudolph *et al., Hum. Pathol.* 32(3):311-319 (2001), as has been aberrant expression products of *mdm2* (Lukas *et al.*, Cancer Res. 61(7):3212-3219 (2001) and cyclin1 and p27 (Porter & Roberts, International Publication WO98/33450, published August 6, 1998).

[0011] The detection of *BRCA1* or *BRCA2* mutations represents a step towards the design of therapies to better control and prevent the appearance of these tumors. Recently, many studies have used gene expression profiling to analyze various cancers, and those studies have provided new diagnosis and prognosis information in the molecular level. *See* Zajchowski *et al.*, "Identification of Gene Expression Profiled that Predict the Aggressive Behavior of Breast Cancer Cells," *Cancer Res.* 61:5168 (2001); West *et al.*, "Predicting the Clinical Status of Human Breast Cancer by Using Gene Expression Profiles," *Proc. Natl. Acad. Sci. U.S.A.* 98:11462 (2001); van 't Veer *et al.*, "Gene Expression Profiling Predicts the Outcome of Breast Cancer," *Nature* 415:530 (2002); Roberts *et al.*, "Diagnosis and Prognosis of Breast Cancer Patients," WO 02/103320; Sorlie *et al., Proc. Natl. Acad. Sci. U.S.A.* 100:8418 (2003); Perou *et al., Nature* 406:747 (2000); Khan *et al., Cancer Res* 58, 5009 (1998); Golub *et al., Science* 286, 531 (1999); DeRisi *et al., Nat. Genet.* 14:457 (1996); Alizadeh *et al., Nature* 403, 503 (2000). Methods for the identification of informative genesets for various cancers have also been described. *See* Roberts *et al.*, "Diagnosis and Prognosis of Breast Cancer Patients," WO 02/103320; Golub *et al.*, United States Patent No. 6,647,341.

[0012] Genesets have been identified that are informative for differentiating individuals having, or suspected of having, breast cancer based on estrogen receptor (ER) status, or *BRCA1* mutation vs. sporadic (*i.e.*, other than *BRCA1*-type) mutation status. *See* Roberts *et al.*, WO 02/103320; van't Veer *et al., Nature* 415:530 (2001). Genesets have also been identified that enable the classification of sporadic tumor-type individuals as those who will likely have no metastases within five years of initial diagnosis (*i.e.*, individuals with a good prognosis) or those who will likely have a metastasis within five years of initial diagnosis (*i.e.*, those having a poor prognosis). Roberts, *supra*; van't Veer, *supra*.

[0013] Roberts *et al.* WO 02/103320 describes a 70-gene set, useful for the prognosis of breast cancer, which outperformed clinical measures of prognosis, and which showed good potential in selecting good outcome patients, thereby avoiding over-treatment. van de Vijver *et al., N. Engl. J. Med.* 347:1999 (2002). The expression of genes with most predictive value, however, were not homogeneous among poor patients, suggesting the need for improvement.

[0014] Although the patterns of gene expression as described in Roberts *et al.* were correlated with existing clinical indicators such as estrogen receptor and *BRCA1* status, clinical measures were not incorporated. Furthermore, although the poor-outcome group in particular showed heterogeneity in expression pattern, the best classifier decision rule found during these studies was a fairly simple one based on the similarity of a patient profile to the average profile of a good-outcome training group.

[0015] Because it is evident that breast cancer is the result of more than one type of molecular event, there still exists a need for improved prognostic methods so that appropriate courses of therapy may be provided. Genesets having improved prognostic power can be identified by first identifying discrete subsets of breast cancer patients, and then identifying genesets informative for prognosis within those subsets of patients. Individuals having breast cancer, or who are suspected of having breast cancer, would then be provided therapies appropriate to the molecular mechanisms underlying the cancer. The present invention provides such methods for breast cancer, and for other cancers, diseases or conditions.

## 3. SUMMARY OF THE INVENTION

[0016] The present invention provides methods of identifying relevant subsets of conditions, and the identification of markers relevant to those subsets, for example, for prognosis of individuals classifiable into one of those subsets. The invention further provides sets of markers useful for the prognosis of individuals having breast cancer, wherein those patients have been classified according to one or more characteristics of breast cancer.

[0017] Thus, the present invention provides a method of identifying a set of informative genes or markers for a condition comprising a plurality of phenotypic or genotypic characteristics, comprising: (a) classifying each of a plurality of samples or individuals on the basis of one or more phenotypic or genotypic characteristics of said condition into a plurality of first classes; and (b) identifying within each of said first classes a first set of genes or markers informative for said condition, wherein said first set of genes or markers within each of said first classes is unique to said class relative to other first classes. In a specific embodiment, this method further comprises additionally classifying into a plurality of second classes said samples or individuals in at least one of said first classes on the basis of a phenotypic or genotypic characteristic different that that used in said classifying step (a); and identifying within at least one of said second classes a second set of informative genes or markers, wherein said second set of informative genes or markers within each of said second classes is unique to said second class relative to other first and second classes.

[0018] The invention further provides a method of identifying a set of informative genes or markers for a condition comprising a plurality of phenotypic or genotypic characteristics, comprising: (a) classifying each of a plurality of samples or individuals on the basis of one or more phenotypic or genotypic characteristics into a plurality of first classes; (b) classifying at least one of said first classes into a plurality of second classes on the basis of phenotypic or genotypic characteristic different than that used in said classifying step (a); and (c) identifying within at least one of said first classes or said second classes a set of genes or markers informative for said condition, wherein said second set of genes or markers is unique to said class relative to other first and second classes.

[0019] The invention further provides a method of identifying a set of informative genes or markers for a condition comprising a plurality of phenotypic or genotypic characteristics, comprising: (a) selecting a first characteristic from said plurality of phenotypic or genotypic characteristics; (b) identifying at least two first condition classes differentiable by said first characteristic; (c) selecting a plurality of individuals classifiable into at least one of said first condition classes; and (d) identifying in samples derived from each of said plurality of individuals a set of genes or markers informative for said condition within said at least one of said first condition classes.

[0020] The invention further provides a method of classifying an individual with a condition as having a good prognosis or a poor prognosis, comprising: (a) classifying said individual into one of a plurality of patient classes, said patient classes being differentiated by one or more phenotypic, genotypic or clinical characteristics of said condition; (b) determining the level of expression of a plurality of genes or their encoded proteins in a cell sample taken from the individual relative to a control, said plurality of genes or their encoded proteins comprising genes or their encoded proteins informative for prognosis of the patient class into which said individual is classified; and (c) classifying said individual as having a good prognosis or a poor prognosis on the basis of said level of expression. In a specific embodiment, said condition is cancer, said good prognosis is the non-occurrence of metastases within five years of initial diagnosis, and said poor prognosis is the occurrence of metastases within five years of initial diagnosis. In a more specific embodiment, said cancer is breast cancer. In another specific embodiment, said control is the average level of expression of each of said plurality of genes or their encoded proteins across a plurality of samples derived from individuals identified as having a poor prognosis. In a more specific embodiment, said classifying step (c) is carried out by a method comprising comparing the level of expression of each of said plurality of genes or their encoded proteins to said average

level of expression of each corresponding gene or its encoded protein in said control, and classifying said individual as having a poor prognosis if said level of expression correlates with said average level of expression of each of said genes or their encoded proteins in said control more strongly than would be expected by chance. In another specific embodiment, said control is the average level of expression of each of said plurality of genes or their encoded proteins across a plurality of samples derived from individuals identified as having a good prognosis. In a more specific embodiment, said classifying in step (c) is carried out by a method comprising comparing the level expression of each of said plurality of genes or their encoded proteins to said average level of expression of each corresponding gene or its encoded protein in said control, and classifying said individual as having a good prognosis if said level of expression correlates with said average level of expression of each of said genes or their encoded proteins in said control more strongly than would be expected by chance. In another specific embodiment, said plurality of patient classes comprises ER$^-$, *BRCA1* individuals; ER$^-$, sporadic individuals; ER+, ER/AGE high individuals; ER+, ER/AGE low, LN+ individuals; and ER+, ER/AGE low, LN$^-$ individuals.

[0021] The invention further provides a method of classifying a breast cancer patient as having a good prognosis or a poor prognosis comprising: (a) classifying said breast cancer patient as ER$^-$, *BRCA1*; ER$^-$, sporadic; ER+, ER/AGE high; ER+, ER/AGE low, LN+; or ER+, ER/AGE low, LN$^-$; (b) determining the level of expression of a first plurality of genes in a cell sample taken from said breast cancer patient relative to a control, said first plurality of genes comprising two of the genes corresponding to the markers in Table 1 if said breast cancer patient is classified as ER$^-$, *BRCA1*; in Table 2 if said breast cancer patient is classified as ER$^-$ sporadic; in Table 3 if said breast cancer patient is classified as ER+, ER/AGE high; in Table 4 if said breast cancer patient is classified as ER+. ER/AGE low, LN+; or in Table 5 if said breast cancer patient is classified as ER+, ER/AGE low, LN$^-$; and (c) classifying said breast cancer patient as having a good prognosis or a poor prognosis on the basis of the level of expression of said first plurality of genes, wherein said breast cancer patient is "ER/AGE high" if the ratio of the $\log_{10}$(ratio) of ER gene expression to age exceeds a predetermined value, and "ER/AGE low" if the ratio of the $\log_{10}$(ratio) of ER gene expression to age does not exceed said predetermined value. In a specific embodiment, said control is the average level of expression of each of said plurality of genes in a plurality of samples derived from ER$^-$, *BRCA1* individuals, if said breast cancer patient is ER$^-$, *BRCA1*; the average level of expression of each of said plurality of genes in a plurality of samples derived from ER$^-$, sporadic individuals if said breast cancer patient is ER$^-$, sporadic; the

average level of expression of each of said plurality of genes in a plurality of samples derived from ER+, ER/AGE high individuals, if said breast cancer patient is ER+, ER/AGE high; the average level of expression of each of said plurality of genes in a plurality of samples derived from ER+, ER/AGE low, LN+ individuals where said breast cancer patient is ER+, ER/AGE low, LN+; or the average level of expression of each of said plurality of genes in a plurality of samples derived from ER+, ER/AGE low, $LN^-$ individuals where said breast cancer patient is ER+, ER/AGE low, $LN^-$. In a more specific embodiment, each of said individuals has a poor prognosis. In another more specific embodiment, each of said individuals has a good prognosis. In an even more specific embodiment, said classifying step (c) is carried out by a method comprising comparing the level of expression of each of said plurality of genes or their encoded proteins in a sample from said breast cancer patient to said control, and classifying said breast cancer patient as having a poor prognosis if said level of expression correlates with said average level of expression of the corresponding genes or their encoded proteins in said control more strongly than would be expected by chance. In another specific embodiment, said predetermined value of ER is calculated as $ER = 0.1(AGE - 42.5)$, wherein AGE is the age of said individual. In another specific embodiment, said individual is $ER^-$, *BRCA1*, and said plurality of genes comprises two of the genes for which markers are listed in Table 1. In another specific embodiment, said individual is $ER^-$, *BRCA1*, and said plurality of genes comprises all of the genes for which markers are listed in Table 1. In another specific embodiment, said individual is $ER^-$, sporadic, and said plurality of genes comprises two of the genes for which markers are listed in Table 2. said individual is $ER^-$, sporadic, and said plurality of genes comprises all of the genes for which markers are listed in Table 2. In another specific embodiment, said individual is ER+, ER/AGE high, and said plurality of genes comprises two of the genes for which markers are listed in Table 3. said individual is ER+, ER/AGE high, and said plurality of genes comprises all of the genes for which markers are listed in Table 3. In another specific embodiment, said individual is ER+, ER/AGE low, LN+, and said plurality of genes comprises two of the genes for which markers are listed in Table 4. In another specific embodiment, said individual is ER+, ER/AGE low, LN+, and said plurality of genes comprises all of the genes for which markers are listed in Table 4. In another specific embodiment, said individual is ER+, ER/AGE low, $LN^-$, and said plurality of genes comprises two of the genes for which markers are listed in Table 4. In another specific embodiment, said individual is ER+, ER/AGE low, $LN^-$, and said plurality of genes comprises all of the genes for which markers are listed in Table 4. In another specific embodiment, the method further comprises determining in said cell sample the level of

expression, relative to a control, of a second plurality of genes for which markers are not found in Tables 1-5, wherein said second plurality of genes is informative for prognosis.

[0022] In another embodiment, the invention provides a method for assigning an individual to one of a plurality of categories in a clinical trial, comprising: (a) classifying said individual as $ER^-$, *BRCA1*, $ER^-$, sporadic; ER+, ER/AGE high; ER+, ER/AGE low, LN+; or ER+, ER/AGE low, $LN^-$; (b) determining for said individual the level of expression of at least two genes for which markers are listed in Table 1 if said individual is classified as $ER^-$, *BRCA1*; Table 2 if said individual is classified as $ER^-$, sporadic; Table 3 if said individual is classified as ER+, ER/AGE high; Table 4 if said individual is classified as ER+, ER/AGE low, LN+; or Table 5 if said individual is classified as ER+, ER/AGE low, $LN^-$; (c) determining whether said individual has a pattern of expression of said at least two genes that correlates with a good prognosis or a poor prognosis; and (d) assigning said individual to one category in a clinical trial if said individual has a good prognosis, and assigning said individual to a second category in said clinical trial if said individual has a poor prognosis. In a specific embodiment, said individual is additionally assigned to a category in said clinical trial on the basis of the classification of said individual as determined in step (a). In another specific embodiment, said individual is additionally assigned to a category in said clinical trial on the basis of any other clinical, phenotypic or genotypic characteristic of breast cancer. In another specific embodiment, said method further comprises determining in said cell sample the level of expression, relative to a control, of a second plurality of genes for which markers are not found in Tables 1-5, wherein said second plurality of genes is informative for prognosis of breast cancer, and determining from the expression of said second plurality of genes, in addition to said first plurality of genes, whether said individual has a good prognosis or a poor prognosis.

[0023] A microarray comprising probes complementary and hybridizable to a plurality of the genes for which markers are listed in any of Tables 1-5. The invention further provides a microarray comprising probes complementary and hybridizable to a plurality of the genes for which markers are listed in Table 1, each of the genes for which markers are listed in Table 1, a plurality of the genes for which markers are listed in Table 2, each of the genes for which markers are listed in Table 2, a plurality of the genes for which markers are listed in Table 3, each of the genes for which markers are listed in Table 3, a plurality of the genes for which markers are listed in Table 4, each of the genes for which markers are listed in Table 4, a plurality of the genes for which markers are listed in Table 5, or each of the genes for which markers are listed in Table 5. The invention further provides any one of the above

microarrays, wherein said probes are at least 50% of the probes on said microarray. The invention further provides any one of the above microarrays, wherein said probes are at least 90% of the probes on said microarray. The invention further provides microarray comprising probes complementary and hybridizable to a plurality of the genes for which markers are listed in any of Tables 1-5, wherein said probes are complementary and hybridizable to at least 75% of the genes for which markers are listed in Table 1; are complementary and hybridizable to at least 75% of the genes for which markers are listed in Table 2; are complementary and hybridizable to at least 75% of the genes for which markers are listed in Table 3; are complementary and hybridizable to at least 75% of the genes for which markers are listed in Table 4; and are complementary and hybridizable to at least 75% of the genes for which markers are listed in Table 5, wherein said probes, in total, are at least 50% of the probes on said microarray.

[0024] The invention further comprises a kit comprising any one of the above microarrays in a sealed container.

[0025] The invention further provides a method of identifying a set of genes informative for a condition, said condition having a plurality of phenotypic or genotypic characteristics such that samples may be categorized by at least one of said phenotypic or genotypic characteristics into at least one characteristic class, said method comprising: (a) selecting a plurality of samples from individuals having said condition; (b) identifying a first set of genes informative for said characteristic class using said plurality of samples; (c) predicting the characteristic class of each of said plurality of samples; (d) discarding samples for which said characteristic class is incorrectly predicted; (e) repeating steps (c) and (d) at least once; and (f) identifying a second set of genes informative for said characteristic class using samples in said plurality of samples remaining after step (e).

[0026] The invention further provides a method for assigning an individual to one of a plurality of categories in a clinical trial, comprising: (a) classifying the individual into one of a plurality of condition categories differentiated by at least one genotypic or phenotypic characteristic of the condition; (b) determining the level of expression, in a sample derived from said individual, of a plurality of genes informative for said condition category; (c) determining whether said level of expression of said plurality of genes indicates that the individual has a good prognosis or a poor prognosis; and (d) assigning the individual to a category in a clinical trial on the basis of prognosis.

4. BRIEF DESCRIPTION OF THE DRAWINGS

[0027] FIG. 1 depicts the decision tree that resulted in the five patient subsets used to identify informative prognosis-related genes.

[0028] FIG. 2: Relationship between ER level and age. (A) Scatter plot of ER vs. age for ER+ patients. Black dots indicate metastases free samples, and gray dots indicate metastases samples. It appears that patients of ER+ group can be subdivided into "ER+, ER/AGE high" group (above the black line) and "ER+, ER/AGE low" (below the black line) group. The black line is approximated by ER = 0.1*(AGE-42.5), and the dashed line by ER = 0.1*(age-50). Within each population, the ER level also increases with age. (B) Age distribution of all patients in ER+ samples. A bimodal distribution is observed. (C) ER-modulated age (age – 10*) distribution of all patients in ER+ samples. A bimodal distribution is observed. (D) Age distribution of samples with metastasis. (E) ER-modulated age distribution of samples with metastasis. The three peaks appearing in this distribution suggest a polymorphism.

[0029] FIG. 3. Performance of classifier for the "$ER^-$/sporadic" group. (A) Error rate obtained from leave-one-out cross validation (LOOCV) for predicting the disease outcome as a function of the number of reporter genes used in the classifier. (B) Scatter plot between correlation to good group (X axis) and to poor group (Y axis). Circles indicate metastases-free samples, squares indicate samples with metastases. Dashed line: threshold for separating poor from good. (C) Error rate calculated with respect to good outcome group (good outcome misclassified as poor divided by total number of good), or poor outcome group (poor outcome misclassified as good divided by total number of poor), or the average of the two rates.

[0030] FIG. 4. Performance of classifier for the "ER+, ER/AGE high" group. (A) Error rate obtained from leave-one-out cross validation (LOOCV) for predicting the disease outcome as a function of the number of reporter genes used in the classifier. (B) Scatter plot between correlation to good group (X axis) and to poor group (Y axis). Circles indicate metastases-free samples, and squares indicate samples with metastases. Dashed line: threshold for separating poor from good. (C) Error rate calculated with respect to good outcome group (good outcome misclassified as poor divided by total number of good), or poor outcome group (poor outcome misclassified as good divided by total number of poor), or the average of the two rates.

[0031] FIG. 5. Performance of classifier for the "ER+, ER/AGE low/$LN^-$" group. (A) Error rate obtained from leave-one-out cross validation (LOOCV) for predicting the disease outcome as a function of the number of reporter genes used in the classifier. (B) Scatter plot

between correlation to good group (X axis) and to poor group (Y axis). Circles indicate metastases-free samples, and squares indicates samples with metastases. Dashed line indicates the threshold for separating poor from good. (C) Error rate calculated with respect to good outcome group (good outcome misclassified as poor divided by total number of good), or poor outcome group (poor outcome misclassified as good divided by total number of poor), or the average of the two rates.

[0032] FIG. 6. Performance of classifier for the "ER+, ER/AGE low/LN+" group. (A) Error rate obtained from leave-one-out cross validation (LOOCV) for predicting the disease outcome as a function of the number of reporter genes used in the classifier. (B) Scatter plot between correlation to good group (X axis) and to poor group (Y axis). Circles indicate metastases free samples, squares indicate samples with metastases. Dashed line: threshold for separating poor from good. (C) Error rate calculated with respect to good outcome group (good outcome misclassified as poor divided by total number of good), or poor outcome group (poor outcome misclassified as good divided by total number of poor), or the average of the two rates.

[0033] FIG. 7. Performance of classifier for the "$ER^-$, *BRCA1*" group. (A) Error rate obtained from leave-one-out cross validation (LOOCV) for predicting the disease outcome as a function of the number of reporter genes used in the classifier. (B) Scatter plot between correlation to good group (X axis) and to poor group (Y axis). Circles indicate metastases free samples, squares indicate samples with metastases. Dashed line: threshold for separating poor from good. (C) Error rate calculated with respect to good outcome group (good outcome misclassified as poor divided by total number of good), or poor outcome group (poor outcome misclassified as good divided by total number of poor), or the average of the two rates.

[0034] FIG. 8. Heatmaps of genes representing key biological functions in subgroups of patients: A: Cell cycle genes are predictive of outcome in patients with ER/age high. B: Cell cycle genes are not predictive of outcome in "ER- and sporadic" patients C: Glycolysis genes are predictive of outcome in patients with ER/age low and LN-. D: Glycolysis genes are not predictive of outcome in 'ER- & BRCA1" patients.

## 5. DETAILED DESCRIPTION OF THE INVENTION

### 5.1 INTRODUCTION

[0035] The present invention provides methods for classifying individuals having a condition, such as a disease, into at least one subset of that condition, wherein the subset is

defined by one or more phenotypic or genotypic characteristics of the condition. Such individuals may be eukaryotes or prokaryotes, may be non-human animals such as mammals, for example humans, primates, rodents, felines, canines, etc.; birds, reptiles, fish, etc. "Individuals" as used herein also encompasses single-celled organisms, or colonies thereof, such as bacteria and yeast. The condition may be a disease, such as cancer, and may be a specific cancer, such as breast cancer. The present invention provides methods of determining the prognosis of individuals having a condition, such as cancer, for example, breast cancer, or who are suspected of having the condition, by the use of a combination of clinical parameters and gene expression pattern data. In the example of breast cancer, patient groups are first classified according to at least one of age, lymph node (LN) status, estrogen receptor (ER) level, and *BRCA1* mutation status into discrete patient subsets. These clinical factors have been implicated in tumor etiology as well as differences in disease outcome. The differences in gene expression or in tumor fate related to these parameters likely represent differences in tumor origin and tumor genesis, and are therefore good candidates for tumor stratification. Genesets informative for prognosis within each subset are then identified. New breast cancer patients are then classified using the same criteria, and a prognosis is made based on the geneset specific for the patient subset into which the patient falls.

[0036] In the process of constructing prognosis classifier within each subset, particular attention is paid to the homogeneous patterns related to the tumor outcome. Emergence of such homogeneous prognosis patterns may indicate the most common mechanism to metastasis within a subset. At the same time, successful identification of such patterns also justifies the parameters being used for the tumor stratification. To differentiate this approach from an mRNA-alone approach, we refer to the current approach of integrating clinical data with the gene expression data as a "comprehensive prognosis".

## 5.2 DEFINITIONS

[0037] As used herein, "*BRCA1* tumor" or "*BRCA1* type" means a tumor having cells containing a mutation of the *BRCA1* locus.

[0038] The "absolute amplitude" of correlation means the distance, either positive or negative, from a zero value; *i.e.*, both correlation coefficients -0.35 and 0.35 have an absolute amplitude of 0.35.

[0039]

[0040] "Marker" means a cellular constituent, or a modification of a cellular constituent (*e.g.*, an entire gene, EST derived from that gene, a protein encoded by that gene, post-translational modification of the protein, etc.) the expression or level of which changes between certain conditions. Where a change in a characteristic of the constituent correlates with a certain condition, the constituent is a marker for that condition.

[0041] "Marker-derived polynucleotides" means the RNA transcribed from a marker gene, any cDNA or cRNA produced therefrom, and any nucleic acid derived therefrom, such as synthetic nucleic acid having a sequence derived from the gene corresponding to the marker gene.

[0042] A "similarity value" is a number that represents the degree of similarity between two things being compared. For example, a similarity value may be a number that indicates the overall similarity between a patient's expression profile using specific phenotype-related markers and a control specific to that phenotype (for instance, the similarity to a "good prognosis" template, where the phenotype is a good prognosis). The similarity value may be expressed as a similarity metric, such as a correlation coefficient, or may simply be expressed as the expression level difference, or the aggregate of the expression level differences, between a patient sample and a template.

[0043] A "patient subset" is a group of individuals, all of whom have a particular condition, that is distinguished from other individuals having that condition by one or more phenotypic, genotypic or clinical characteristics of the condition. For example, where the condition is breast cancer, individuals may belong to an "$ER^+$" or an "$ER^-$" patient subset, or may belong to a particular age group patient subset.

[0044] A gene and/or marker is "informative" for a condition, phenotype, genotype or clinical characteristic if the expression of the gene or marker is correlated or anticorrelated with the condition, phenotype, genotype or clinical characteristic to a greater degree than would be expected by chance.

[0045] An individual is classified as "ER/AGE high" if the ratio of the log(ratio) of ER expression to the age of individual for which the ER expression level is determined is equal to or greater than a predetermined threshold value, and the individual is classified as "ER/AGE low" if the ratio is less than the threshold value. In one embodiment, the threshold value is calculated as ER = 0.1 (AGE - 42.5); thus, the threshold for a 45-year old individual in this embodiment is 0.1 (45-42.5), or 0.25. Thus, in this embodiment, if the log(ratio) of ER expression in the individual is equal to or greater than 0.25, the individual is classified as "ER/AGE high"; otherwise, the individual is classified as "ER/AGE low."

### 5.3 IDENTIFICATION OF DIAGNOSTIC AND PROGNOSTIC MARKER SETS

#### 5.3.1 IDENTIFICATION OF CONDITION SUBSETS

[0046] The present invention provides methods of identifying of sets of genes and/or markers useful in the diagnosis and prognosis of breast cancer. More generally, the invention also provides methods of identifying sets of genes and/or markers useful in the diagnosis or prognosis of other cancers, and even more generally, of identifying sets of genes and/or markers useful in the differentiation between subgroups of individuals having a particular condition, such as a disease.

[0047] The method may be applied to any condition for which a plurality of phenotypic or genotypic subsets may be identified. The condition may be a disease; for example, the condition may be cancer, an autoimmune disease, an inflammatory disease, an infectious disease, a neurological disease, a degenerative disease, etc. The condition may be environmental; for example, the condition may be a particular diet, geographic location, etc.; the condition may be exposure to a compound, including, for example, a drug, a toxin, a carcinogen, a foodstuff, a poison, an inhaled compound, an ingested compound, etc.; the condition may be a particular genetic background or predisposition to a medical condition; etc.

[0048] Where the condition is cancer, the condition may be any cancer, for example, without limitation: leukemias, including acute leukemia, acute lymphocytic leukemia, acute myelocytic leukemia, myeloblastic leukemia, promyelocytic leukemia, myelomonocytic leukemia, monocytic leukemia, and erythroleukemia; chronic leukemia, such as chronic myelocytic (granulocytic) leukemia or chronic lymphocytic leukemia; polycythemia vera; lymphomas, such as Hodgkin's disease and non-Hodgkin's disease; multiple myeloma; Waldenström's macroglobulinemia; heavy chain disease; solid tumors, such as sarcomas and carcinomas, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder

carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, melanoma, neuroblastoma, or retinoblastoma; etc.

[0049] Rather than stratifying individuals, such as patients or tumor samples derived from patients, by gene expression patterns in the first instance, however, the method of identifying sets of genes informative for a condition begins by identifying phenotypic, genotypic or clinical subsets of individuals within the larger class of individuals having or affected by the condition.

[0050] In one embodiment, the condition is cancer, and the subsets are distinguished by phenotypes, genotypes, and/or clinical characteristics of the cancer. In this embodiment, groups of individuals are classified according to one or more phenotypes, genotypes, or clinical characteristics relevant to the cancer into patient subsets. At any step in the process of subdividing a patient population into patient subsets, the level expression of one or more genes may be determined in order to identify whether a prognosis-informative set of genes may be identified for the particular patient subset. If an informative gene set is identified, but is not as informative as desired, the patient subset may be divided and a new geneset identified. These subsets may be further subdivided. For example, a group of individuals affected by a particular cancer may be classified first on the basis of a phenotypic, genotypic or clinical characteristic A into subsets S1 and S2. The level of expression is then determined of a plurality of genes in tumor samples taken from individuals that fall within subsets S1 or S2 in order to identify sets of genes informative for prognosis within these subsets. Subsets S1 and S2 may then be subdivided into two or more subsets each based on other phenotypic, genotypic or clinical characteristics. The basis for subdivision, if performed, need not be the same for both S1 and S2. For example, in various embodiments, S1 is not subdivided, while S2 is subdivided on the basis of characteristic B; S1 is subdivided based on characteristic B while S2 is not subdivided; S1 and S2 are both subdivided on the basis of characteristic B; S1 is subdivided based on characteristic B, while S2 is subdivided according to characteristic C; and so on. For a particular decision matrix leading to a plurality of patient subsets, the preferred outcome is a prognosis-informative set of genes for each patient subset. Different decision matrices may lead to different patient subsets, which, in turn, may result in different sets of prognosis-informative genes.

[0051] In the specific example of breast cancer, a plurality of phenotypes, genotypes or clinical indications are used to classify a patient as being a member of one of a plurality of patient subsets, wherein the subsets are medically, biochemically or genetically relevant to

breast cancer. For example, a group of patients may be classified into patient subsets based on criteria including, but not limited to, estrogen receptor (ER) status, type of tumor (*i.e.*, *BRCA1*-type or sporadic), lymph node status, grade of cancer, invasiveness of the tumor, or age. "BRCA1-type" indicates that the *BRCA1* mutation is present. In each classification step, a group of cancer patients may be classified into only two classes, for example, ER+ or $ER^{-}$, or into three or more subsets (for example, by tumor grade), depending upon the characteristic used to determine the subsets. As used herein, "ER+" indicates that the estrogen receptor is expressed at some level; for example, it may indicate that the estrogen receptor is detectably expressed, or may indicate that more than 10% of cells may be histologically stained for the receptor, etc. Conversely, "ER–" indicates that the estrogen receptor is expressed at a reduced level or not at all; for example, it may indicate that the receptor is not detectably expressed, or that 10% or less of cells may be histologically stained for the receptor, etc. Marker gene sets optimized for each phenotypic class are preferably determined after the subsets are established. Where informative markers for a particular patient subset, distinguished from another subset by a particular characteristic of the condition of interest, cannot be determined, the subset may be further divided by another characteristic of the condition to create a plurality of second patient subsets, whereupon genes informative for these second patient subsets may be identified.

[0052] FIG. 1 depicts the process, described in the Examples, of subdivision of a collection of breast cancer patients according to phenotypic and genotypic characteristics relevant to breast cancer, in preparation for identification of genes informative for prognosis. A collection of breast cancer tumor samples was first subdivided by estrogen receptor status. ER status was chosen because the presence or absence of the estrogen receptor greatly influences the expression of other genes. In the ER+ patient subset, it was noted that patients appeared to be bimodally distributed by ER level vs. age; that is, ER level dependence upon age tended to fall within two classes, as separated by the solid line in FIG. 2A. This bimodality was used to further subdivide ER+ individuals into "ER+, ER/AGE high" individuals and "ER+, ER/AGE low" individuals. A set of informative genes was identified for the ER+, ER/AGE high patient subset. An informative set was not identified for the ER+, ER/AGE low subset, however, so the subset of patients was further divided into LN+ and LN– individuals. Thus, in one embodiment, the present invention provides a method of identifying a set of informative genes or markers for a condition comprising a plurality of phenotypic or genotypic characteristics, comprising (a) classifying each of a plurality of samples or individuals on the basis of one phenotypic or genotypic characteristic into a

plurality of first classes; and (b) identifying within each of said first classes a set of informative genes or markers, wherein said set of informative genes or markers within each said first classes is unique to said class.

### 5.3.2 IDENTIFICATION OF MARKER SETS INFORMATIVE FOR PATIENT SUBSETS

[0053] Once a patient subset is identified, markers, such as genes, informative for a particular condition, such as prognosis, may be identified. In one embodiment, the method for identifying marker sets is as follows. This example describes the use of genes and gene-derived nucleic acids as markers; however, proteins or other cellular constituents may be used as markers of the condition.

[0054] After extraction and labeling of target polynucleotides, the expression of a plurality of markers (genes) in a sample X is compared to the expression of the plurality markers in a standard or control. In one embodiment, the standard or control comprises target polynucleotide molecules derived from a sample from a normal individual (*i.e.*, an individual not afflicted with breast cancer). In a preferred embodiment, the standard or control is a pool of target polynucleotide molecules. The pool may be derived from collected samples from a number of normal individuals. In a preferred embodiment, the pool comprises samples taken from a number of individuals having sporadic-type tumors. In another preferred embodiment, the pool comprises an artificially-generated population of nucleic acids designed to approximate the level of nucleic acid derived from each marker found in a pool of marker-derived nucleic acids derived from tumor samples. In yet another embodiment, the pool is derived from normal or breast cancer cell lines or cell line samples. In a preferred embodiment, the pool comprises samples taken from individuals within a specific patient subset, *e.g.*, "ER+, ER/AGE high" individuals, wherein each of said individuals has a good prognosis, or each of said individuals has a poor prognosis. Of course, where, for example, expressed proteins are used as markers, the proteins are obtained from the individual's sample, and the standard or control could be a pool of proteins from a number of normal individuals, or from a number of individuals having a particular state of a condition, such as a pool of samples from individuals having a particular prognosis of breast cancer.

[0055] The comparison may be accomplished by any means known in the art. For example, expression levels of various markers may be assessed by separation of target polynucleotide molecules (*e.g.*, RNA or cDNA) derived from the markers in agarose or polyacrylamide gels, followed by hybridization with marker-specific oligonucleotide probes. Alternatively, the comparison may be accomplished by the labeling of target polynucleotide molecules

followed by separation on a sequencing gel. Polynucleotide samples are placed on the gel such that patient and control or standard polynucleotides are in adjacent lanes. Comparison of expression levels is accomplished visually or by means of densitometer. In a preferred embodiment, the expression of all markers is assessed simultaneously by hybridization to a microarray. In each approach, markers meeting certain criteria are identified as informative for the prognosis of breast cancer.

[0056] Marker genes are selected based upon significant difference of expression in a condition, such as a disease, as compared to a standard or control condition. Marker genes may be screened, for example, by determining whether they show significant variation within a set of samples of interest. Genes that do not show a significant amount of variation within the set of samples are presumed not to be informative for the disease or condition, and are not selected as markers for the disease or condition. Genes showing significant variation within the sample set are candidate informative genes for the disease or condition. The degree of variation may be estimated by calculating the standard deviation of the expression of the gene, or ratio of expression between sample and control, within the set of samples. The expression, or ratio of expressions, may be transformed by any means, *e.g.*, linear or log transformation. Selection may be made based upon either significant up- or down regulation of the marker in the patient sample. Selection may also be made by calculation of the statistical significance (*i.e.*, the p-value) of the correlation between the expression of the marker and the disease and condition. Preferably, both selection criteria are used. Thus, in one embodiment of the present invention, markers associated with prognosis of breast cancer within a patient subset are selected where the markers show both more than two-fold change (increase or decrease) in expression as compared to a standard, and the p-value for the correlation between the existence of breast cancer and the change in marker expression is no more than 0.01 (*i.e.*, is statistically significant).

[0057] In the context of the present invention, "good prognosis" indicates a desired outcome for a particular condition, especially a particular disease, and "poor prognosis" indicates an undesired outcome of the condition. For example, where the condition is cancer, a "good prognosis" may mean partial or complete remission, and "poor prognosis" may mean reappearance of the cancer after treatment. In the specific example of breast cancer, "good prognosis" means the likelihood of non-reoccurrence of metastases within a period of 1, 2, 3, 4, 5 or more years after initial diagnosis, and "poor prognosis" means the likelihood of reoccurrence of metastasis within that period. In a more specific example, "good prognosis"

means the likelihood of non-reoccurrence of metastases within 5 years after initial diagnosis, and "poor prognosis" means the likelihood of reoccurrence of metastasis within that period.

[0058] In a more specific embodiment for cancer, for example, breast cancer, using a number of breast cancer tumor samples, markers are identified by calculation of correlation coefficients $\rho$ between the clinical category or clinical parameter(s) $\vec{c}$ and the linear, logarithmic or any transform of the expression ratio $\vec{r}$ across all samples for each individual gene. Specifically, the correlation coefficient may be calculated as:

$$\rho = (\vec{c} \bullet \vec{r}) / (\|\vec{c}\| \cdot \|\vec{r}\|). \qquad \text{Equation (1)}$$

[0059] Markers for which the coefficient of correlation exceeds a cutoff are identified as prognosis-informative markers specific for a particular clinical type, *e.g.*, good prognosis, within a given patient subset. Such a cutoff or threshold may correspond to a certain significance of discriminating genes obtained by Monte Carlo simulations. The threshold depends upon the number of samples used; the threshold can be calculated as 3 X $1/\sqrt{n-3}$, where $1/\sqrt{n-3}$ is the distribution width and $n$ = the number of samples. In a specific embodiment, markers are chosen if the correlation coefficient is greater than about 0.3 or less than about -0.3.

[0060] Next, the significance of the correlation is calculated. This significance may be calculated by any statistical means by which such significance is calculated. In a specific example, a set of correlation data is generated using a Monte-Carlo technique to randomize the association between the expression difference of a particular marker and the clinical category. The frequency distribution of markers satisfying the criteria in the Monte-Carlo runs is used to determine whether the number of markers selected by correlation with clinical data is significant.

[0061] Once a marker set is identified, the markers may be rank-ordered in order of significance of discrimination. One means of rank ordering is by the amplitude of correlation between the change in gene expression of the marker and the specific condition being discriminated. Another, preferred, means is to use a statistical metric. In a specific embodiment, the metric is a t-test-like statistic:

$$t = \left(\langle x_1 \rangle - \langle x_2 \rangle\right) \Big/ \sqrt{\left[\sigma_1^2 (n_1 - 1) + \sigma_2^2 (n_2 - 1)\right] / (n_1 + n_2 - 1) / \left(1/n_1 + 1/n_2\right)} \qquad \text{Equation (2)}$$



CAJD: 362883.2

[0062] In this equation, $\langle x_1 \rangle$ is the error-weighted average of the log ratio of transcript expression measurements within a first clinical group (*e.g.*, good prognosis), $\langle x_2 \rangle$ is the error-weighted average of log ratio within a second, related clinical group (*e.g.*, poor prognosis), $\sigma_1$ is the variance of the log ratio within the first clinical group (*e.g.*, good prognosis), $n_1$ is the number of samples for which valid measurements of log ratios are available, $\sigma_2$ is the variance of log ratio within the second clinical group (*e.g.*, poor prognosis), and $n_2$ is the number of samples for which valid measurements of log ratios are available. The *t*-value represents the variance-compensated difference between two means.

[0063] The rank-ordered marker set may be used to optimize the number of markers in the set used for discrimination. This is accomplished generally in a "leave one out" method as follows. In a first run, a subset, for example five, of the markers from the top of the ranked list is used to generate a template, where out of X samples, X-1 are used to generate the template, and the status of the remaining sample is predicted. This process is repeated for every sample until every one of the X samples is predicted once. In a second run, additional markers, for example five additional markers, are added, so that a template is now generated from 10 markers, and the outcome of the remaining sample is predicted. This process is repeated until the entire set of markers is used to generate the template. For each of the runs, type 1 error (false negative) and type 2 errors (false positive) are counted; the optimal number of markers is that number where the type 1 error rate, or type 2 error rate, or preferably the total of type 1 and type 2 error rate is lowest.

[0064] For prognostic markers, validation of the marker set may be accomplished by an additional statistic, a survival model. This statistic generates the probability of tumor distant metastases as a function of time since initial diagnosis. A number of models may be used, including Weibull, normal, log-normal, log logistic, log-exponential, or log-Rayleigh (Chapter 12 "Life Testing", S-PLUS 2000 GUIDE TO STATISTICS, Vol. 2, p. 368 (2000)). For the "normal" model, the probability of distant metastases P at time *t* is calculated as

$$P = \alpha \times \exp\left(-t^2/\tau^2\right) \qquad \text{Equation (3)}$$

where $\alpha$ is fixed and equal to 1, and $\tau$ is a parameter to be fitted and measures the "expected lifetime".

[0065] It is preferable that the above marker identification process be iterated one or more times by excluding one or more samples from the marker selection or ranking (*i.e.*, from the calculation of correlation). Those samples being excluded are the ones that can not be

predicted correctly from the previous iteration. Preferably, those samples excluded from marker selection in this iteration process are included in the classifier performance evaluation, to avoid overstating the performance.

[0066] It will be apparent to those skilled in the art that the above methods, in particular the statistical methods, described above, are not limited to the identification of markers associated with the prognosis of breast cancer within a particular patient subset, but may be used to identify set of marker genes associated with any phenotype. The phenotype can be the presence or absence of a disease such as cancer, or the presence or absence of any identifying clinical condition associated with that cancer. In the disease context, the phenotype may be a prognosis such as a survival time, probability of distant metastases of a disease condition, or likelihood of a particular response to a therapeutic or prophylactic regimen. The phenotype need not be cancer, or a disease; the phenotype may be a nominal characteristic associated with a healthy individual.

[0067] Thus, the invention provides a method of identifying a set of informative genes or markers for a condition comprising a plurality of phenotypic of genotypic characteristics, comprising: (a) classifying each of a plurality of samples or individuals on the basis of one or more phenotypic or genotypic characteristics of said condition into a plurality of first classes; (b) identifying within each of said first classes a first set of genes or markers informative for said condition, wherein said first set of genes or markers within each of said first classes is unique to said class relative to other classes. In a specific embodiment, samples or individuals in at least one of said first classes are additionally classified on the basis of a phenotypic or genotypic characteristic different that that used to distinguish said first classes. and identifying within at least one of said second classes a second set of informative genes or markers, wherein said second set of informative genes or markers within each of said second classes is unique to said second class relative to other classes. In another embodiment, the invention provides a method of identifying a set of informative genes or markers for a condition comprising a plurality of phenotypic of genotypic characteristics, comprising: (a) classifying each of a plurality of samples or individuals on the basis of one or more phenotypic or genotypic characteristics into a plurality of first classes; (b) classifying at least one of said first classes into a plurality of second classes on the basis of phenotypic or genotypic characteristic different than that used to distinguish said plurality of first classes; (c) identifying within at least one of said first classes or said second classes a set of genes or markers informative for said condition, wherein said second set of genes or markers is unique to said class relative to other classes. The invention further provides a method of identifying

a set of informative genes or markers for a condition comprising a plurality of phenotypic or genotypic characteristics, comprising: (a) selecting a first characteristic from said plurality of phenotypic or genotypic characteristics; (b) identifying at least two first condition classes differentiable by said first characteristic; (c) selecting a plurality of individuals classifiable into at least one of said first condition classes; and (d) identifying in samples derived from each of said plurality of individuals a set of genes or markers informative for said condition within said at least one of said first condition classes.

### 5.3.3 CLASSIFIER GENESETS FOR FIVE PATIENT SUBSETS

[0068] The present invention provides sets of markers useful for the prognosis of breast cancer. The markers were identified according to the above methods in specific subsets of individuals with breast cancer. Generally, the marker sets were identified within a population of breast cancer patients that had been first stratified into five phenotypic categories based on criteria relevant to breast cancer prognosis, including estrogen receptor (ER) status, lymph node status, type of mutation(s) (*i.e.*, BRCA1-type or sporadic) and age at diagnosis. More specifically, patients, and tumors from which samples were taken, were classified as $ER^-$, sporadic (*i.e.*, being both estrogen receptor negative and having a non-*BRCA1*-type tumor); $ER^-$, *BRCA1* (*i.e.*, being both estrogen receptor negative and having a *BRCA1*-type tumor); ER+, ER/AGE high (*i.e.*, estrogen receptor positive with a high ratio of the log (ratio) of estrogen receptor gene expression to age); ER+, ER/AGE low, LN+ (*i.e.*, estrogen receptor positive with a low ratio of the log (ratio) of estrogen receptor gene expression to age, lymph node positive); and $ER^+$, ER/AGE low, $LN^-$ (*i.e.*, estrogen receptor positive with a low ratio of the log (ratio) of estrogen receptor gene expression to age, lymph node negative). The rationale for subdivision of the original patient set into these five subsets is detailed in the Examples (Section 6). The marker sets useful for each of the subsets above are provided in Tables 1-5, respectively.

Table 1: Geneset of 20 markers used to classify $ER^-$, sporadic individuals.

| Accession/ Contig No. | Gene | Avg good xdev | Avg poor xdev | Corre-lation | Description | Sp_xref_keyword_list |
|---|---|---|---|---|---|---|
| AF055033 | IGFBP5 | -2.12 | 0.88 | 0.54 | insulin-like growth factor binding protein 5 | Growth factor binding, Glycoprotein, Signal, 3D-structure |
| NM_000599 | IGFBP5 | -3.41 | 0.43 | 0.53 | insulin-like growth factor binding protein 5 | Growth factor binding, Glycoprotein, Signal, 3D-structure |
| L27560 | IGFBP5 | -4.55 | 0 | 0.52 | EST | Hypothetical protein |
| AF052162 | FLJ12443 | -0.27 | 1.6 | 0.52 | EST | Hypothetical protein |

| Accession/ Contig No. | Gene | Avg good xdev | Avg poor xdev | Corre-lation | Description | Sp_xref_keyword_list |
|---|---|---|---|---|---|---|
| NM_001456 | FLNA | -0.61 | 2.47 | 0.52 | filamin A, alpha (actin binding protein 280) | Hypothetical protein, Actin-binding, Phosphorylation, Repeat, Polymorphism, Disease mutation |
| NM_002205 | ITGA5 | -0.37 | 2.08 | 0.49 | integrin, alpha 5 (fibronectin receptor, alpha polypeptide) | Integrin, Cell adhesion, Receptor, Glycoprotein, Transmembrane, Signal, Calcium, Repeat |
| NM_013261 | PPARGC1 | 0.09 | 1.54 | 0.47 | peroxisome proliferative activated receptor, gamma, coactivator 1 | |
| NM_001605 | AARS | 0.39 | 2.36 | 0.51 | alanyl-tRNA synthetase | Aminoacyl-tRNA synthetase, Protein biosynthesis, Ligase, ATP-binding |
| X87949 | HSPA5 | -0.03 | 2.03 | 0.49 | heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa) | ATP-binding, Hypothetical protein, Endoplasmic reticulum, Signal |
| Contig50950_RC | NGEF | -1.17 | 3.2 | 0.52 | neuronal guanine nucleotide exchange factor | |
| NM_005689 | ABCB6 | -0.51 | 2.26 | 0.48 | ATP-binding cassette, sub-family B (MDR/TAP), member 6 | ATP-binding, Transport, Transmembrane, Mitochondrion, Inner membrane, Transit peptide, Hypothetical protein |
| NM_004577 | PSPH | -0.56 | 3.05 | 0.51 | phosphoserine phosphatase | Hydrolase, Serine biosynthesis, Magnesium, Phosphorylation |
| NM_003832 | PSPHL | -2.08 | 2.18 | 0.5 | phosphoserine phosphatase-like | |
| NM_002422 | MMP3 | -0.96 | 2.54 | 0.5 | matrix metalloproteinase 3 (stromelysin 1, progelatinase) | Hydrolase, Metalloprotease, Glycoprotein, Zinc, Zymogen, Calcium, Collagen degradation, Extracellular matrix, Signal, Polymorphism, 3D-structure |
| Contig37562_RC | | -3.42 | -6.02 | -0.59 | ESTs | |
| NM_018465 | MDS030 | -0.82 | -3.28 | -0.58 | uncharacterized hematopoietic stem/progenitor cells protein MDS030 | Hypothetical protein |
| Contig54661_RC | | -0.79 | -2.08 | -0.54 | ESTs | |
| AB032969 | KIAA1143 | -0.6 | -2.85 | -0.53 | KIAA1143 protein | Hypothetical protein |
| Contig55353_RC | KIAA1915 | -0.27 | -1.82 | -0.47 | KIAA1915 protein | Hypothetical protein |
| NM_005213 | CSTA | 2.11 | -3.4 | -0.49 | cystatin A (stefin A) | Thiol protease inhibitor, 3D-structure |

Table 2. Geneset of 10 markers used to classify $ER^-$, *BRCA1* individuals.

| Accession/ Contig No. | Gene | Avg good xdev | Avg poor xdev | Correl-ation | Sequence name | Description | Sp_xref_keyword_list |
|---|---|---|---|---|---|---|---|



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| | | | | | | | |
|---|---|---|---|---|---|---|---|
| AF005487 | | 6.08 | 0.5 | -0.79 | HLA-DRB6 | Homo sapiens MHC class II antigen (DRB6) mRNA, HLA-DRB6*0201 allele, sequence. | MHC |
| Contig50728_RC | | 4.02 | 0.25 | -0.77 | | ESTs, Weakly similar to S26650 DNA-binding protein 5 - human [H.sapiens] | |
| Contig53598_RC | | 8.41 | 3.26 | -0.77 | FLJ11413 | hypothetical protein FLJ11413 | Hypothetical protein |
| NM_002888 | RARRES1 | 6.9 | 0.05 | -0.87 | RARRES1 | retinoic acid receptor responder (tazarotene induced) 1 | Receptor, Transmembrane, Signal-anchor |
| NM_005218 | DEFB1 | 5.14 | -3.02 | -0.81 | DEFB1 | defensin, beta 1 | Antibiotic, Signal, 3D-structure |
| U17077 | BENE | 2.72 | -1.72 | -0.77 | BENE | BENE protein | Transmembrane |
| Contig14683_RC | | 1.29 | -2.31 | -0.74 | | ESTs | |
| Contig53641_RC | | -3.29 | 4.23 | 0.75 | MAGE-E1 | MAGE-E1 protein | Hypothetical protein |
| Contig56678_RC | | -6.7 | -9.73 | -0.82 | | ESTs, Highly similar to THYA_HUMAN Prothymosin alpha [H.sapiens] | |
| NM_005461 | KRML | 0.88 | -3.38 | -0.75 | MAFB | v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian) | Transcription regulation, Repressor, DNA-binding, Nuclear protein, Hypothetical protein |

Table 3. Geneset of 50 markers used to classify ER+, ER/AGE high individuals.

| Accession/ Contig No. | Gene | Avg good xdev | Avg poor xdev | Corre-lation | Description | Sp_xref_keyword_list |
|---|---|---|---|---|---|---|
| NM_003600 | STK15 | -2.93 | 2.08 | 0.8 | serine/threonine kinase 6 | ATP-binding, Kinase, Serine/threonine-protein kinase, Transferase |
| NM_003158 | STK6 | -1.57 | 1.42 | 0.78 | serine/threonine kinase 6 | ATP-binding, Kinase, Serine/threonine-protein kinase, Transferase |
| NM_007019 | UBCH10 | -2.98 | 2.62 | 0.81 | ubiquitin-conjugating enzyme E2C | Hypothetical protein, Ubl conjugation pathway, Ligase, Multigene family, Mitosis, Cell cycle, Cell division |
| NM_013277 | ID-GAP | -2.43 | 2.43 | 0.77 | Rac GTPase activating protein 1 | Hypothetical protein |
| NM_004336 | BUB1 | -2.04 | 1.39 | 0.77 | BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast) | Transferase, Serine/threonine-protein kinase, ATP-binding, Cell cycle, Nuclear protein, Mitosis, Phosphorylation, Polymorphism |
| NM_006607 | PTTG2 | -1.71 | 1.49 | 0.72 | pituitary tumor-transforming 2 | |
| AK001166 | FLJ11252 | -1.33 | 0.99 | 0.71 | hypothetical protein FLJ11252 | Hypothetical protein |
| NM_004701 | CCNB2 | -4.62 | 2.01 | 0.81 | cyclin B2 | Cyclin, Cell cycle, Cell division, Mitosis |

| Accession/ Contig No. | Gene | Avg good xdev | Avg poor xdev | Corre-lation | Description | Sp_xref_keyword_list |
|---|---|---|---|---|---|---|
| Contig57584_RC | | -3.68 | 2.04 | 0.78 | likely ortholog of mouse gene rich cluster, C8 gene | |
| NM_006845 | KNSL6 | -4.13 | 1.05 | 0.73 | kinesin-like 6 (mitotic centromere-associated kinesin) | Hypothetical protein, Motor protein, Microtubules, ATP-binding, Coiled coil, Nuclear protein |
| Contig38901_RC | | -3.08 | 1.15 | 0.75 | hypothetical protein MGC45866 | Hypothetical protein |
| NM_018410 | DKFZp762E1312 | -4.38 | 1.49 | 0.75 | hypothetical protein DKFZp762E1312 | Hypothetical protein |
| NM_003981 | PRC1 | -3.52 | 2.17 | 0.78 | protein regulator of cytokinesis 1 | |
| NM_001809 | CENPA | -5.04 | 0.98 | 0.75 | centromere protein A, 17kDa | Hypothetical protein, Chromosomal protein, Nuclear protein, DNA-binding, Centromere, Antigen |
| NM_003504 | CDC45L | -2.67 | 1.22 | 0.73 | CDC45 cell division cycle 45-like (S. cerevisiae) | DNA replication, Cell cycle, Nuclear protein, Cell division |
| Contig41413_RC | | -5.43 | 2.15 | 0.74 | ribonucleotide reductase M2 polypeptide | Oxidoreductase, DNA replication, Iron |
| NM_004217 | STK12 | -2.17 | 0.73 | 0.72 | serine/threonine kinase 12 | Hypothetical protein, ATP-binding, Kinase, Serine/threonine-protein kinase, Transferase |
| NM_002358 | MAD2L1 | -2.65 | 2.27 | 0.83 | MAD2 mitotic arrest deficient-like 1 (yeast) | Cell cycle, Mitosis, Nuclear protein, 3D-structure |
| NM_014321 | ORC6L | -2.73 | 1.8 | 0.75 | origin recognition complex, subunit 6 homolog-like (yeast) | Hypothetical protein, DNA replication, Nuclear protein, DNA-binding |
| NM_012291 | KIAA0165 | -1.52 | 1.55 | 0.71 | extra spindle poles like 1 (S. cerevisiae) | Hypothetical protein |
| NM_004203 | PKMYT1 | -3.64 | 2.2 | 0.7 | retinoblastoma-like 2 (p130) | ATP-binding, Kinase, Serine/threonine-protein kinase, Transferase, Transcription regulation, DNA-binding, Nuclear protein, Cell cycle, Phosphorylation, Anti-oncogene |
| M96577 | E2F1 | -2.14 | 1.42 | 0.75 | E2F transcription factor 1 | Transcription regulation, Activator, DNA-binding, Nuclear protein, Phosphorylation, Cell cycle, Apoptosis, Polymorphism |
| NM_002266 | KPNA2 | -3.77 | 1.78 | 0.71 | karyopherin alpha 2 (RAG cohort 1, importin alpha 1) | Transport, Protein transport, Repeat, Nuclear protein, Polymorphism |
| Contig31288_RC | | -2.63 | 0.7 | 0.68 | ESTs, Weakly similar to hypothetical protein | |

| Accession/ Contig No. | Gene | Avg good xdev | Avg poor xdev | Corre- lation | Description | Sp_xref_keyword_list |
|---|---|---|---|---|---|---|
| | | | | | FLJ20489 [Homo sapiens] [H.sapiens] | |
| NM_014501 | E2-EPF | -1.55 | 1.93 | 0.7 | ubiquitin carrier protein | Ubl conjugation pathway, Ligase, Multigene family |
| NM_001168 | BIRC5 | -5.76 | 2.01 | 0.78 | baculoviral IAP repeat-containing 5 (survivin) | Apoptosis, Thiol protease inhibitor, Alternative splicing, 3D-structure, Hypothetical protein, Protease, Receptor |
| NM_003258 | TK1 | -4.57 | 1.38 | 0.71 | thymidine kinase 1, soluble | Transferase, Kinase, DNA synthesis, ATP-binding |
| NM_001254 | CDC6 | -2.46 | 0.28 | 0.72 | CDC6 cell division cycle 6 homolog (S. cerevisiae) | ATP-binding, Cell division |
| NM_004900 | DJ742C19 .2 | -2.96 | 0.13 | 0.69 | apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B | Hydrolase |
| NM_004702 | CCNE2 | -3.12 | 2.13 | 0.81 | cyclin E2 | Cell cycle, Cell division, Cyclin, Hypothetical protein, Phosphorylation, Alternative splicing, Nuclear protein |
| AL160131 | | -3.07 | 2.42 | 0.7 | hypothetical protein MGC861 | Hypothetical protein |
| NM_016359 | LOC5120 3 | -3.22 | 2.61 | 0.76 | nucleolar protein ANKT | Hypothetical protein, Nuclear protein |
| NM_004856 | KNSL5 | -1.52 | 1.1 | 0.71 | kinesin-like 5 (mitotic kinesin-like protein 1) | Motor protein, Cell division, Microtubules, ATP-binding, Coiled coil, Mitosis, Cell cycle, Nuclear protein |
| NM_000057 | BLM | -1.54 | 0.76 | 0.71 | Bloom syndrome | Hydrolase, Helicase, ATP-binding, DNA-binding, Nuclear protein, DNA replication, Disease mutation |
| NM_018455 | BM039 | -2.44 | 1.18 | 0.7 | uncharacterized bone marrow protein BM039 | |
| NM_002106 | H2AFZ | -2.49 | 1.53 | 0.72 | H2A histone family, member Z | Chromosomal protein, Nucleosome core, Nuclear protein, DNA-binding, Multigene family |
| Contig64688 | | -2.68 | 3.1 | 0.73 | hypothetical protein FLJ23468 | Hypothetical protein |
| Contig44289_RC | | -1.65 | 1.6 | 0.67 | ESTs | |
| Contig28552_RC | | -1.37 | 1.53 | 0.68 | diaphanous homolog 3 (Drosophila) | Hypothetical protein, Coiled coil, Repeat, Alternative splicing |
| Contig46218_RC | | -1.31 | 1.56 | 0.68 | ESTs, Weakly similar to T19201 hypothetical protein C11G6.3 - Caenorhabditis elegans [C. elegans] | |
| Contig28947_RC | | -1.3 | 0.98 | 0.67 | cell division cycle 25A | Hypothetical protein, Cell division, Mitosis, Hydrolase, Alternative |

| Accession/ Contig No. | Gene | Avg good xdev | Avg poor xdev | Corre- lation | Description | Sp_xref_keyword_list |
|---|---|---|---|---|---|---|
| | | | | | | splicing, Multigene family, 3D-structure |
| NM_016095 | LOC51659 | -1.4 | 2.13 | 0.67 | HSPC037 protein | Hypothetical protein |
| NM_003090 | SNRPA1 | -3.26 | 0.95 | 0.7 | small nuclear ribonucleoprotein polypeptide A' | Hypothetical protein, Nuclear protein, RNA-binding, Ribonucleoprotein, Leucine-rich repeat, Repeat, 3D-structure |
| NM_002811 | PSMD7 | -2.48 | 1.89 | 0.7 | proteasome (prosome, macropain) 26S subunit, non-ATPase, 7 (Mov34 homolog) | Proteasome |
| Contig38288_RC | | -2.34 | 0.97 | 0.67 | hypothetical protein DKFZp762A2013 | Hypothetical protein |
| NM_003406 | YWHAZ | -1.5 | 2.79 | 0.68 | tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide | Brain, Neurone, Phosphorylation, Acetylation, Multigene family, 3D-structure |
| AL137540 | NTN4 | 2.13 | -4.61 | -0.69 | netrin 4 | Hypothetical protein, Laminin EGF-like domain, Signal |
| AL049367 | | 1.9 | -3.2 | -0.68 | EST | Transducer, Prenylation, Lipoprotein, Multigene family, Acetylation |
| NM_013409 | FST | 1.04 | -5.78 | -0.69 | follistatin | Glycoprotein, Repeat, Signal, Alternative splicing |
| NM_000060 | BTD | 3.1 | -1.45 | -0.67 | biotinidase | Hydrolase, Glycoprotein, Signal, Disease mutation |

Table 4. Geneset of 50 markers used to classify ER+, ER/AGE low, LN+ individuals.

| Accession/ Contig No. | Gene | Avg good xdev | Avg poor xdev | Correl- ation | Description | Sp_xref_keyword_list |
|---|---|---|---|---|---|---|
| NM_006417 | MTAP44 | -1.5 | 3 | 0.69 | Fc fragment of IgG, low affinity IIb, receptor for (CD32) | Hydrolase, Hypothetical protein, Immunoglobulin domain, IgG-binding protein, Receptor, Transmembrane, Glycoprotein, Signal, Repeat, Multigene family, Polymorphism, NAD, One-carbon metabolism, Serine protease, Zymogen, Protease, Alternative splicing, Chromosomal translocation, Proto-oncogene, Galaptin, |

| Accession/ Contig No. | Gene | Avg good xdev | Avg poor xdev | Correl-ation | Description | Sp_xref_keyword_list |
|---|---|---|---|---|---|---|
| | | | | | | Lectin, Antigen |
| NM_006820 | GS3686 | -4.3 | 4.06 | 0.69 | chromosome 1 open reading frame 29 | Hypothetical protein |
| NM_001548 | IFIT1 | -3.4 | 4.27 | 0.71 | Interferon-induced protein with tetratricopeptide repeats 1 | Repeat, TPR repeat, Interferon induction |
| Contig41538_RC | | -2.5 | 3.16 | 0.68 | ESTs, Moderately similar to hypothetical protein FLJ20489 [*Homo sapiens*] | |
| NM_016816 | OAS1 | -1.7 | 3.29 | 0.75 | 2',5'-oligoadenylate synthetase 1, 40/46kDa | RNA-binding, Transferase, Nucleotidyltransferase, Interferon induction, Alternative splicing |
| Contig51660_RC | | -2.1 | 2.65 | 0.66 | 28kD interferon responsive protein | Transmembrane |
| Contig43645_RC | | -4.8 | 1.44 | 0.63 | *Homo sapiens*, clone IMAGE:4428577, mRNA, partial cds | Hypothetical protein |
| AF026941 | | -4.6 | 2.71 | 0.63 | EST, Weakly similar to 2004399A chromosomal protein [*Homo sapiens*] | Hypothetical protein |
| NM_007315 | STAT1 | -3.5 | 1.8 | 0.59 | signal transducer and activator of transcription 1, 91kDa | Transcription regulation, DNA-binding, Nuclear protein, Phosphorylation, SH2 domain, Alternative splicing, 3D-structure |
| NM_002038 | G1P3 | -4.1 | 5.64 | 0.79 | interferon, alpha-inducible protein (clone IFI-6-16) | Interferon induction, Transmembrane, Signal, Alternative splicing |
| NM_005101 | ISG15 | -5.6 | 5.34 | 0.77 | interferon-stimulated protein, 15 kDa | Interferon induction, Repeat |
| NM_002462 | MX1 | -6.1 | 0.83 | 0.56 | myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse) | Hypothetical protein, Interferon induction, GTP-binding, Multigene family, Antiviral |
| NM_005532 | IFI27 | -5.8 | 2.81 | 0.59 | interferon, alpha-inducible protein 27 | Interferon induction, Transmembrane |
| NM_002346 | LY6E | -2.1 | 3.58 | 0.75 | lymphocyte antigen 6 complex, locus E | Signal, Antigen, Multigene family, Membrane, GPI-anchor |
| NM_016817 | OAS2 | -3.6 | 1.89 | 0.59 | 2'-5'-oligoadenylate synthetase 2, 69/71kDa | RNA-binding, Transferase, Nucleotidyltransferase, Repeat, Interferon induction, Alternative splicing, Myristate |
| Contig44909_RC | | -2.3 | 1.13 | 0.55 | hypothetical protein BC012330 | Hypothetical protein |
| NM_017414 | USP18 | -4.1 | 3.37 | 0.72 | ubiquitin specific protease 18 | Ubl conjugation pathway, Hydrolase, Thiol protease, Multigene family |
| NM_004029 | IRF7 | -2.4 | 3.67 | 0.66 | interferon regulatory | Collagen, Transcription |

| Accession/ Contig No. | Gene | Avg good xdev | Avg poor xdev | Correl-ation | Description | Sp_xref_keyword_list |
|---|---|---|---|---|---|---|
| | | | | | factor 7 | regulation, DNA-binding, Nuclear protein, Activator, Alternative splicing |
| NM_004335 | BST2 | -3.2 | 3.22 | 0.57 | bone marrow stromal cell antigen 2 | Transmembrane, Glycoprotein, Signal-anchor, Polymorphism |
| NM_002759 | PRKR | -2.4 | 1.8 | 0.58 | protein kinase, interferon-inducible double stranded RNA dependent | Transferase, Serine/threonine-protein kinase, ATP-binding, Repeat, Phosphorylation, Interferon induction, RNA-binding, 3D-structure |
| NM_006332 | IFI30 | -3.8 | 2.65 | 0.64 | interferon, gamma-inducible protein 30 | Oxidoreductase, Interferon induction, Glycoprotein, Lysosome, Signal, Hypothetical protein |
| NM_009587 | LGALS9 | -3.2 | 2.08 | 0.6 | lectin, galactoside-binding, soluble, 9 (galectin 9) | Galaptin, Lectin, Repeat, Alternative splicing |
| NM_003641 | IFITM1 | -2.4 | 5.54 | 0.63 | interferon induced transmembrane protein 1 (9-27) | Interferon induction, Transmembrane |
| NM_017523 | HSXIAPAF1 | -1 | 2.84 | 0.7 | XIAP associated factor-1 | Hypothetical protein |
| NM_014314 | RIG-I | -1.3 | 3.55 | 0.62 | RNA helicase | ATP-binding, Helicase, Hydrolase, Hypothetical protein |
| Contig47563_RC | | -2.2 | 3.11 | 0.56 | ESTs | |
| AI497657_RC | | -4.4 | 5.61 | 0.74 | guanine nucleotide binding protein 4 | Transducer, Prenylation, Lipoprotein, Multigene family |
| NM_000735 | CGA | -4.3 | 2.5 | 0.58 | glycoprotein hormones, alpha polypeptide | Hormone, Glycoprotein, Signal, 3D-structure |
| NM_004988 | MAGEA1 | -1.4 | 6.31 | 0.64 | melanoma antigen, family A, 1 (directs expression of antigen MZ2-E) | Antigen, Multigene family, Polymorphism, Tumor antigen |
| Contig54242_RC | | -1.2 | 4.1 | 0.65 | chromosome 17 open reading frame 26 | Hypothetical protein |
| NM_004710 | SYNGR2 | -1.4 | 3.01 | 0.54 | synaptogyrin 2 | Transmembrane |
| NM_001168 | BIRC5 | -3.7 | 3.39 | 0.64 | baculoviral IAP repeat-containing 5 (survivin) | Hypothetical protein, Protease, Receptor, Apoptosis, Thiol protease inhibitor, Alternative splicing, 3D-structure |
| Contig41413_RC | | -4.4 | 2.61 | 0.57 | ribonucleotide reductase M2 polypeptide | Oxidoreductase, DNA replication, Iron |
| NM_004203 | PKMYT1 | -3.4 | 3.79 | 0.6 | retinoblastoma-like 2 (p130) | ATP-binding, Kinase, Serine/threonine-protein kinase, Transferase, Transcription regulation, DNA-binding, Nuclear |

| Accession/ Contig No. | Gene | Avg good xdev | Avg poor xdev | Correl-ation | Description | Sp_xref_keyword_list |
|---|---|---|---|---|---|---|
| | | | | | | protein, Cell cycle, Phosphorylation, Anti-oncogene |
| Contig48913_RC | | -3.1 | 1.72 | 0.55 | *Homo sapiens*, Similar to hypothetical protein PRO1722, clone MGC:15692 IMAGE:3351479, mRNA, complete cds | |
| NM_005804 | DDXL | -2.5 | 1.42 | 0.58 | DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 39 | ATP-binding, Helicase, Hydrolase, Hypothetical protein |
| NM_016359 | LOC51203 | -1.7 | 3.6 | 0.57 | nucleolar protein ANKT | Hypothetical protein, Nuclear protein |
| NM_001645 | APOC1 | -2.9 | 3.43 | 0.58 | apolipoprotein C-I | Plasma, Lipid transport, VLDL, Signal, 3D-structure, Polymorphism |
| Contig37895_RC | | -2 | 2.05 | 0.55 | ESTs | |
| NM_005749 | TOB1 | -1.3 | 4.96 | 0.59 | transducer of ERBB2, 1 | Phosphorylation |
| NM_000269 | NME1 | -1.3 | 2.98 | 0.55 | non-metastatic cells 1, protein (NM23A) expressed in | Transferase, Kinase, ATP-binding, Nuclear protein, Anti-oncogene, Disease mutation |
| NM_014462 | LSM1 | -1 | 4.5 | 0.57 | Lsm1 protein | Nuclear protein, Ribonucleoprotein, mRNA splicing, mRNA processing, RNA-binding |
| Contig31221_RC | | -1.4 | 3.83 | 0.56 | HTPAP protein | |
| NM_005326 | HAGH | -1.9 | 4.29 | 0.57 | hydroxyacyl glutathione hydrolase | Hydrolase, Zinc, 3D-structure |
| Contig42342_RC | | 0.78 | -3.2 | -0.6 | *Homo sapiens* cDNA FLJ39417 fis, clone PLACE6016942 | Hypothetical protein |
| AL137540 | NTN4 | 2.24 | -3.9 | -0.6 | netrin 4 | Laminin EGF-like domain, Signal, Hypothetical protein |
| Contig40434_RC | | 1.64 | -5.6 | -0.6 | wingless-type MMTV integration site family, member 5A | Developmental protein, Glycoprotein, Signal |
| Contig1632_RC | | 1.03 | -3.9 | -0.6 | hypothetical protein MGC17921 | Hypothetical protein |
| NM_014246 | CELSR1 | 0.95 | -4.6 | -0.6 | cadherin, EGF LAG seven-pass G-type receptor 1 (flamingo homolog, *Drosophila*) | G-protein coupled receptor, Transmembrane, Glycoprotein, EGF-like domain, Calcium-binding, Laminin EGF-like domain, Repeat, Developmental protein, Hydroxylation, Signal, Alternative splicing, Hypothetical protein |
| NM_005139 | ANXA3 | 1.26 | -6.2 | -0.6 | annexin A3 | Annexin, Calcium/phospholipid-binding, Repeat, Phospholipase A2 inhibitor, 3D-structure, |

| Accession/ Contig No. | Gene | Avg good xdev | Avg poor xdev | Correl-ation | Description | Sp_xref_keyword_list |
|---|---|---|---|---|---|---|
| | | | | | | Polymorphism |

Table 5. Geneset of 65 markers used to classify ER+, ER/AGE low, LN⁻ individuals.

| Accession/ Contig No. | Gene | Avg good xdev | Avg poor xdev | Correl-ation | Sequence name | Description | Sp_xref_keyword_list |
|---|---|---|---|---|---|---|---|
| M55914 | MPB1 | -2.82 | 1.25 | 0.5 | ENO1 | enolase 1, (alpha) | DNA-binding, Transcription regulation, Repressor, Nuclear protein, Lyase, Glycolysis, Magnesium, Multigene family, Hypothetical protein |
| NM_005945 | MPB1 | -3.06 | 1.19 | 0.49 | ENO1 | Homo sapiens enolase 1, (alpha) (ENO1), mRNA. | Glycolysis, Hypothetical protein, Lyase, Magnesium, DNA-binding, Transcription regulation, Repressor, Nuclear protein, Multigene family |
| NM_001428 | ENO1 | -2.53 | 1.18 | 0.46 | ENO1 | enolase 1, (alpha) | DNA-binding, Transcription regulation, Repressor, Nuclear protein, Lyase, Glycolysis, Magnesium, Multigene family, Hypothetical protein |
| NM_001216 | CA9 | -4.72 | 1.49 | 0.6 | CA9 | carbonic anhydrase IX | Lyase, Zinc, Transmembrane, Glycoprotein, Antigen, Signal, Nuclear protein, Polymorphism |
| NM_001124 | ADM | -5.68 | 2.99 | 0.56 | ADM | adrenomedullin | Hormone, Amidation, Cleavage on pair of basic residues, Signal |
| NM_000584 | IL8 | -2.45 | 2.04 | 0.54 | IL8 | interleukin 8 | Cytokine, Chemotaxis, Inflammatory response, Signal, Alternative splicing, 3D-structure |
| D25328 | PFKP | -4.19 | 3.29 | 0.56 | PFKP | Phosphofructo-kinase, platelet | Kinase, Transferase, Glycolysis, Repeat, Allosteric enzyme, Phosphorylation, Magnesium, Multigene family |
| NM_006096 | NDRG1 | -5.45 | 5.97 | 0.77 | NDRG1 | N-myc downstream regulated gene 1 | Hypothetical protein, Nuclear protein, Repeat |
| NM_004994 | MMP9 | -5.53 | 1.07 | 0.49 | MMP9 | matrix | Hydrolase, |



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| Accession/ Contig No. | Gene | Avg good xdev | Avg poor xdev | Correl-ation | Sequence name | Description | Sp_xref_keyword_list |
|---|---|---|---|---|---|---|---|
| | | | | | | metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase) | Metalloprotease, Glycoprotein, Zinc, Zymogen, Calcium, Collagen degradation, Extracellular matrix, Repeat, Signal, Polymorphism, 3D-structure |
| NM_003311 | TSSC3 | -4.57 | 5.58 | 0.68 | TSSC3 | tumor suppressing subtransferable candidate 3 | |
| NM_006086 | TUBB4 | -5.19 | 2.85 | 0.59 | TUBB4 | tubulin, beta, 4 | G-protein coupled receptor, Transmembrane, Glycoprotein, Phosphorylation, Lipoprotein, Palmitate, Polymorphism, Hypothetical protein, GTP-binding, Receptor, Microtubules, Multigene family |
| NM_006115 | PRAME | -4.48 | 2.77 | 0.61 | PRAME | preferentially expressed antigen in melanoma | Antigen |
| NM_004345 | CAMP | -2.02 | 1.37 | 0.49 | CAMP | cathelicidin antimicrobial peptide | Antibiotic, Signal |
| NM_018455 | BM039 | -2.34 | 0.76 | 0.47 | BM039 | uncharacterized bone marrow protein BM039 | |
| Contig49169_RC | | -1.17 | 1.5 | 0.46 | SUV39H2 | suppressor of variegation 3-9 (Drosophila) homolog 2; hypothetical protein FLJ23414 | Hypothetical protein, Nuclear protein |
| Contig45032_RC | | -1.37 | 0.77 | 0.45 | FLJ14813 | hypothetical protein FLJ14813 | Hypothetical protein, ATP-binding, Kinase, Serine/threonine-protein kinase, Transferase |
| NM_000917 | P4HA1 | -1.54 | 4.31 | 0.62 | P4HA1 | procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide I | Dioxygenase, Collagen, Oxidoreductase, Iron, Vitamin C, Alternative splicing, Glycoprotein, Endoplasmic reticulum, Signal |
| NM_002046 | GAPD | -2.51 | 3.42 | 0.6 | GAPD | glyceraldehyde-3-phosphate dehydrogenase | Glycolysis, NAD, Oxidoreductase, Hypothetical protein, Multigene family |
| NM_000365 | TPI1 | -1.81 | 2.94 | 0.56 | TPI1 | triosephosphate isomerase 1 | Fatty acid biosynthesis, |

| Accession/ Contig No. | Gene | Avg good xdev | Avg poor xdev | Correl-ation | Sequence name | Description | Sp_xref_keyword_list |
|---|---|---|---|---|---|---|---|
| | | | | | | | Gluconeogenesis, Glycolysis, Isomerase, Pentose shunt, Disease mutation, Polymorphism, 3D-structure |
| NM_014364 | GAPDS | -1.08 | 2.88 | 0.58 | GAPDS | glyceraldehyde-3-phosphate dehydrogenase, testis-specific | Glycolysis, Oxidoreductase, NAD |
| NM_005566 | LDHA | -2.01 | 4.01 | 0.59 | LDHA | lactate dehydrogenase A | Oxidoreductase, NAD, Glycolysis, Multigene family, Disease mutation, Polymorphism |
| NM_000291 | PGK1 | -2.28 | 1.68 | 0.51 | PGK1 | phosphoglycerate kinase 1 | Kinase, Transferase, Multigene family, Glycolysis, Acetylation, Disease mutation, Polymorphism, Hereditary hemolytic anemia |
| NM_016185 | LOC51155 | -2.33 | 2.82 | 0.59 | HN1 | hematological and neurological expressed 1 | |
| NM_001168 | BIRC5 | -4.33 | 2.78 | 0.55 | BIRC5 | baculoviral IAP repeat-containing 5 (survivin) | Apoptosis, Thiol protease inhibitor, Alternative splicing, 3D-structure, Hypothetical protein, Protease, Receptor |
| NM_002266 | KPNA2 | -3.75 | 1.34 | 0.47 | KPNA2 | karyopherin alpha 2 (RAG cohort 1, importin alpha 1) | Transport, Protein transport, Repeat, Nuclear protein, Polymorphism |
| Contig31288_RC | | -2.1 | 1.27 | 0.5 | | ESTs, Weakly similar to hypothetical protein FLJ20489 [Homo sapiens] [H.sapiens] | |
| NM_000269 | NME1 | -2.15 | 3.43 | 0.55 | NME1 | non-metastatic cells 1, protein (NM23A) expressed in | Transferase, Kinase, ATP-binding, Nuclear protein, Anti-oncogene, Disease mutation |
| NM_003158 | STK6 | -1.23 | 1.73 | 0.45 | STK6 | serine/threonine kinase 6 | ATP-binding, Kinase, Serine/threonine-protein kinase, Transferase |
| NM_007274 | HBACH | -1.83 | 2.73 | 0.51 | BACH | brain acyl-CoA hydrolase | Hydrolase, Serine esterase, Repeat |
| Contig55188_RC | | -2.36 | 3.28 | 0.47 | FLJ22341 | hypothetical protein FLJ22341 | Hypothetical protein |
| NM_002061 | GCLM | -1.06 | 1.76 | 0.48 | GCLM | glutamate-cysteine ligase, modifier subunit | Ligase, Glutathione biosynthesis |

| Accession/ Contig No. | Gene | Avg good xdev | Avg poor xdev | Correl-ation | Sequence name | Description | Sp_xref_keyword_list |
|---|---|---|---|---|---|---|---|
| NM_004207 | SLC16A3 | -3.11 | 5.07 | 0.67 | SLC16A3 | solute carrier family 16 (monocarboxylic acid transporters), member 3 | Transport, Symport, Transmembrane, Multigene family |
| NM_000582 | SPP1 | -5.09 | 5.47 | 0.53 | SPP1 | secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1) | Hypothetical protein, Glycoprotein, Sialic acid, Biomineralization, Cell adhesion, Phosphorylation, Signal, Alternative splicing |
| NM_001109 | ADAM8 | -2.5 | 3.74 | 0.45 | ADAM8 | a disintegrin and metalloproteinase domain 8 | Hydrolase, Metalloprotease, Zinc, Signal, Glycoprotein, Transmembrane, Antigen |
| D50402 | SLC11A1 | -1.05 | 3.46 | 0.53 | SLC11A1 | solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1 | Transport, Iron transport, Transmembrane, Glycoprotein, Macrophage, Polymorphism |
| AL080235 | DKFZP586E1621 | -1.23 | 1.96 | 0.51 | RIS1 | Ras-induced senescence 1 | Hypothetical protein |
| Contig40552_RC | | -1.26 | 3.96 | 0.54 | FLJ25348 | hypothetical protein FLJ25348 | Hypothetical protein |
| Contig52490_RC | | -0.64 | 3.33 | 0.61 | LOC116238 | hypothetical protein BC014072 | |
| NM_006461 | DEEPEST | -2.1 | 1.85 | 0.46 | SPAG5 | sperm associated antigen 5 | Hypothetical protein |
| Contig56503_RC | | -4.3 | 3.39 | 0.55 | MGC9753 | hypothetical gene MGC9753 | Hypothetical protein |
| Contig63525 | | -1.91 | 3.34 | 0.5 | FLJ13352 | hypothetical protein FLJ13352 | Hypothetical protein |
| NM_001909 | CTSD | -0.83 | 4.6 | 0.51 | CTSD | cathepsin D (lysosomal aspartyl protease) | Hydrolase, Aspartyl protease, Glycoprotein, Lysosome, Signal, Zymogen, Polymorphism, Alzheimer's disease, 3D-structure |
| NM_005063 | SCD | -2.57 | 5.15 | 0.48 | SCD | stearoyl-CoA desaturase (delta-9-desaturase) | Hypothetical protein, Endoplasmic reticulum, Fatty acid biosynthesis, Iron, Oxidoreductase, Transmembrane |
| NM_005165 | ALDOC | -2.43 | 5.02 | 0.48 | ALDOC | aldolase C, fructose-bisphosphate | Lyase, Schiff base, Glycolysis, Multigene family |
| NM_000363 | TNNI3 | -0.54 | 3.58 | 0.48 | TNNI3 | troponin I, cardiac | Hypothetical protein, Muscle protein, Actin-binding, Acetylation, |

| Accession/ Contig No. | Gene | Avg good xdev | Avg poor xdev | Correl-ation | Sequence name | Description | Sp_xref_keyword_list |
|---|---|---|---|---|---|---|---|
| | | | | | | | Disease mutation, Cardiomyopathy, Receptor, Signal |
| AF035284 | | -1.63 | 3.28 | 0.47 | FADS1 | EST | Heme, Hypothetical protein |
| Contig30875_RC | | -0.88 | 3 | 0.6 | | ESTs | |
| NM_018487 | HCA112 | -0.7 | 3.54 | 0.58 | HCA112 | hepatocellular carcinoma-associated antigen 112 | Hypothetical protein |
| NM_001323 | CST6 | -1.63 | 3.84 | 0.57 | CST6 | cystatin E/M | Thiol protease inhibitor, Signal, Glycoprotein |
| NM_006516 | SLC2A1 | -1.66 | 2.22 | 0.46 | SLC2A1 | solute carrier family 2 (facilitated glucose transporter), member 1 | Transmembrane, Sugar transport, Transport, Glycoprotein, Multigene family, Disease mutation |
| NM_007267 | LAK-4P | -1.04 | 3.28 | 0.61 | EVIN1 | expressed in activated T/LAK lymphocytes | Hypothetical protein |
| NM_004710 | SYNGR 2 | -0.84 | 4.81 | 0.56 | SYNGR2 | synaptogyrin 2 | Transmembrane |
| Contig63649_RC | | -1.34 | 6.3 | 0.75 | | ESTs, Weakly similar to 2004399A chromosomal protein [Homo sapiens] [H.sapiens] | |
| NM_003376 | VEGF | -2.12 | 2.42 | 0.46 | VEGF | vascular endothelial growth factor | Hypothetical protein, Mitogen, Angiogenesis, Growth factor, Glycoprotein, Signal, Heparin-binding, Alternative splicing, Multigene family, 3D-structure |
| NM_000799 | EPO | -0.75 | 4.01 | 0.69 | EPO | erythropoietin | Erythrocyte maturation, Glycoprotein, Hormone, Signal, Pharmaceutical, 3D-structure |
| NM_006014 | DXS987 9E | -1.85 | 3.44 | 0.54 | DXS9879E | DNA segment on chromosome X (unique) 9879 expressed sequence | |
| NM_007183 | PKP3 | -0.91 | 4.14 | 0.48 | PKP3 | plakophilin 3 | Cell adhesion, Cytoskeleton, Structural protein, Nuclear protein, Repeat |
| D13642 | SF3B3 | -0.65 | 2.28 | 0.48 | SF3B3 | splicing factor 3b, subunit 3, 130kDa | Hypothetical protein, Spliceosome, mRNA processing, mRNA splicing, Nuclear protein |
| NM_003756 | EIF3S3 | -1.85 | 2.19 | 0.46 | EIF3S3 | eukaryotic translation initiation factor 3, subunit 3 | Initiation factor, Protein biosynthesis |

| Accession/ Contig No. | Gene | Avg good xdev | Avg poor xdev | Correl-ation | Sequence name | Description | Sp_xref_keyword_list |
|---|---|---|---|---|---|---|---|
| | | | | | | gamma, 40kDa | |
| Contig47096_RC | | -0.41 | 4.52 | 0.54 | PFKFB4 | 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 | Kinase, Multifunctional enzyme, Transferase, Hydrolase, ATP-binding, Phosphorylation, Multigene family |
| NM_004209 | SYNGR3 | -0.31 | 3.67 | 0.53 | SYNGR3 | synaptogyrin 3 | Transmembrane |
| Contig3464_RC | | 0.99 | -5.81 | -0.52 | | ESTs | |
| Contig31646_RC | | 1.1 | -7.76 | -0.5 | COL14A1 | collagen, type XIV, alpha 1 (undulin) | Extracellular matrix, Glycoprotein, Hypothetical protein, Collagen, Signal |
| Contig49388_RC | | 1.73 | -1.75 | -0.51 | FLJ13322 | hypothetical protein FLJ13322 | Hypothetical protein |
| Contig41887_RC | | 0.37 | -5.74 | -0.47 | LOC124220 | similar to common salivary protein 1 | Hypothetical protein |

## 5.4 DIAGNOSTIC AND PROGNOSTIC METHODS

### 5.4.1 SAMPLE COLLECTION

[0069] In the present invention, markers, such as target polynucleotide molecules or proteins, are extracted from a sample taken from an individual afflicted with a condition such as breast cancer. The sample may be collected in any clinically acceptable manner, but must be collected such that marker-derived polynucleotides (*i.e.*, RNA) are preserved (if gene expression is to be measured) or proteins are preserved (if encoded proteins are to be measured). For example, mRNA or nucleic acids derived therefrom (*i.e.*, cDNA or amplified DNA) are preferably labeled distinguishably from standard or control polynucleotide molecules, and both are simultaneously or independently hybridized to a microarray comprising some or all of the markers or marker sets or subsets described above. Alternatively, mRNA or nucleic acids derived therefrom may be labeled with the same label as the standard or control polynucleotide molecules, wherein the intensity of hybridization of each at a particular probe is compared. A sample may comprise any clinically relevant tissue sample, such as a tumor biopsy or fine needle aspirate, or a sample of bodily fluid, such as blood, plasma, serum, lymph, ascitic fluid, cystic fluid, urine or nipple exudate. The sample may be taken from a human, or, in a veterinary context, from non-human animals such as ruminants, horses, swine or sheep, or from domestic companion animals such as felines and canines.

[0070] Methods for preparing total and poly(A)+ RNA are well known and are described generally in Sambrook *et al.*, MOLECULAR CLONING - A LABORATORY MANUAL (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989)) and Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, vol. 2, Current Protocols Publishing, New York (1994)).

[0071] RNA may be isolated from eukaryotic cells by procedures that involve lysis of the cells and denaturation of the proteins contained therein. Cells of interest include wild-type cells (*i.e.*, non-cancerous), drug-exposed wild-type cells, tumor- or tumor-derived cells, modified cells, normal or tumor cell line cells, and drug-exposed modified cells. Preferably, the cells are breast cancer tumor cells.

[0072] Additional steps may be employed to remove DNA. Cell lysis may be accomplished with a nonionic detergent, followed by microcentrifugation to remove the nuclei and hence the bulk of the cellular DNA. In one embodiment, RNA is extracted from cells of the various types of interest using guanidinium thiocyanate lysis followed by CsCl centrifugation to separate the RNA from DNA (Chirgwin *et al.*, *Biochemistry* 18:5294-5299 (1979)). Poly(A)+ RNA is selected by selection with oligo-dT cellulose (*see* Sambrook *et al.*, MOLECULAR CLONING - A LABORATORY MANUAL (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989). Alternatively, separation of RNA from DNA can be accomplished by organic extraction, for example, with hot phenol or phenol/chloroform/isoamyl alcohol.

[0073] If desired, RNase inhibitors may be added to the lysis buffer. Likewise, for certain cell types, it may be desirable to add a protein denaturation/digestion step to the protocol.

[0074] For many applications, it is desirable to preferentially enrich mRNA with respect to other cellular RNAs, such as transfer RNA (tRNA) and ribosomal RNA (rRNA). Most mRNAs contain a poly(A) tail at their 3' end. This allows them to be enriched by affinity chromatography, for example, using oligo(dT) or poly(U) coupled to a solid support, such as cellulose or Sephadex™ (see Ausubel et al., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, vol. 2, Current Protocols Publishing, New York (1994). Once bound, poly(A)+ mRNA is eluted from the affinity column using 2 mM EDTA/0.1% SDS.

[0075] The sample of RNA can comprise a plurality of different mRNA molecules, each different mRNA molecule having a different nucleotide sequence. In a specific embodiment, the mRNA molecules in the RNA sample comprise at least 5, 10, 15, 20, 25, 30, 40 or 50 different nucleotide sequences. More preferably, the mRNA molecules of the RNA sample

comprise mRNA molecules corresponding to each of the marker genes. In another specific embodiment, the RNA sample is a mammalian RNA sample.

[0076] In a specific embodiment, total RNA or mRNA from cells are used in the methods of the invention. The source of the RNA can be cells of a plant or animal, human, mammal, primate, non-human animal, dog, cat, mouse, rat, bird, yeast, eukaryote, prokaryote, etc. In specific embodiments, the method of the invention is used with a sample containing total mRNA or total RNA from $1 \times 10^6$ cells or less. In another embodiment, proteins can be isolated from the foregoing sources, by methods known in the art, for use in expression analysis at the protein level.

[0077] Probes to the homologs of the marker sequences disclosed herein can be employed preferably when non-human nucleic acid is being assayed.

[0078] The methods of the invention may employ any molecule suitable as a marker. For example, sets of proteins informative for a particular condition, including a disease, may be determined. As for gene-based markers, levels of variations of different proteins in samples may be determined for phenotypic or genotypic subsets of the condition, and proteins showing significant variation in either level (abundance) or activity, or both, may be identified in order to create a set of proteins informative for one or more of these subsets. Such proteins may be identified, for example, by use of gel electrophoresis, such as one-dimensional polyacrylamide gel electrophoresis, two-dimensional polyacrylamide gel electrophoresis, nondenaturing polyacrylamide gel electrophoresis; isoelectric focusing gels, etc., by use of antibody arrays, etc. Of course, the particular template(s) used to classify the individual depends upon the type(s) of cellular constituents used as markers. For example, where nucleic acids (*e.g.*, genes or nucleic acids derived from expressed genes) are used as markers, the template comprises nucleic acids (or the level of expression or abundance thereof); where proteins are used as markers, the template comprises proteins, for example, the level or abundance of those proteins in a set of individuals; etc.

### 5.4.2 USE OF PROGNOSTIC GENESETS FOR BREAST CANCER

[0079] According to the present invention, once genesets informative for a plurality of subsets of a condition are identified, an individual is classified into one of these subsets and a prognosis is made based on the expression of the genes, or their encoded proteins, in the geneset for that subset in a breast cancer tumor sample taken from the individual.

[0080] For example, a particular hypothetical condition has four relevant phenotypes, A, B and C. In this example, based on these characteristics, genesets informative for prognosis of

four patient subsets A+, B+; A+, B–, C+; A+, B–, C–; and A– are identified by the method described above. Thus, an individual having the condition would first be classified according to phenotypes A-D into one of the four patient subsets. In one embodiment, therefore, the invention provides for the classification of an individual having a condition into one of a plurality of patient subsets, wherein a set of genes informative for prognosis for the subset has been identified. A sample is then taken from the individual, and the expression of the prognostically-informative genes in the sample is analyzed and compared to a control. In various embodiments, the control is the average expression of informative genes in a pool of samples taken from good prognosis individuals classifiable into that patient subset; the average expression of informative genes in a pool of samples taken from poor prognosis individuals classifiable into that patient subset; a set of mathematical values that represent gene expression levels of good prognosis individuals classifiable into that patient subset; etc.

[0081] In a specific embodiment, the condition is breast cancer, and the phenotypic, genotypic and/or clinical classes are: $ER^-$, *BRCA1* individuals; $ER^-$, sporadic individuals; ER+, ER/AGE high individuals; ER+, ER/AGE low, LN+ individuals; and ER+, ER/AGE low, $LN^-$ individuals. In this embodiment, an individual may be classified as ER+ or $ER^-$. If the individual is $ER^-$, the individual is additionally classified as having a *BRCA1*-type or sporadic tumor. $ER^-$ individuals are thus classified as $ER^-$, *BRCA1* or $ER^-$, sporadic. Alternatively, if the individual is classified as ER+, the individual is additionally classified as having a high or low ratio of the log (ratio) of the level of expression of the gene encoding the estrogen receptor to the individual's age. Individuals having a low ratio are additionally classified as LN+ or LN–. ER+ individuals are thus classified as ER+, ER/AGE high; ER+, ER/AGE low, LN+, or ER+, ER/AGE low, $LN^-$. Of course, the individual's ER status, tumor type, age and LN status may be identified in any order, as long as the individual is classified into one of these five subsets.

Thus, in one embodiment, the invention provides a method of classifying an individual with a condition as having a good prognosis or a poor prognosis, comprising: (a) classifying said individual into one of a plurality of patient classes, said patient classes being differentiated by one or more phenotypic, genotypic or clinical characteristics of said condition; (b) determining the level of expression of a plurality of genes or their encoded proteins in a cell sample taken from the individual relative to a control, said plurality of genes or their encoded proteins comprising genes or their encoded proteins in a cell sample taken from the individual relative to a control, said plurality of genes or their encoded proteins comprising genes or their encoded proteins informative for prognosis of the patient class into

which said individual is classified; and (c) classifying said individual as having a good prognosis or a poor prognosis on the basis of said level of expression. In a specific embodiment, said condition is breast cancer, said good prognosis is the non-occurrence of metastases within five years of initial diagnosis, and said poor prognosis is the occurrence of metastases within five years of initial diagnosis. In an more specific embodiment, said classifying said individual with a condition as having a good prognosis or a poor prognosis is carried out by comparing the level expression of each of said plurality of genes or their encoded proteins to said average level of expression of each corresponding gene or its encoded protein in said control, and classifying said individual as having a good prognosis poor prognosis if said level of expression correlates with said average level of expression of each of said genes or their encoded proteins in a good prognosis control or a poor prognosis control, respectively, more strongly than would be expected by chance. In a more specific embodiment of the method, said plurality of patient subsets comprises $ER^-$, *BRCA1* individuals; $ER^-$, sporadic individuals; ER+, ER/AGE high individuals; ER+, ER/AGE low, LN+ individuals; and ER+, ER/AGE low, $LN^-$ individuals. In another embodiment, said control is the average level of expression of each of said plurality of genes informative for prognosis in a pool of tumor samples from individuals classified into said subset who have a good prognosis or good outcome, or who have a poor prognosis or good outcome. In another specific embodiment, said control is a set of mathematical values representing the average level of expression of genes informative for prognosis in tumor samples of individuals classifiable into said subset who have a good prognosis, or who have a poor prognosis.

[0082] It is evident that the different patient subsets described herein reflect different molecular mechanisms of the initiation of tumor formation and metastasis. Thus, the genesets listed in tables 1-5 are also useful for diagnosing a person as having a particular type of breast cancer in the first instance. Thus, the invention also provides a method of diagnosing an individual as having a particular subtype of breast cancer, comprising determining the level of expression in a sample from said individual of a plurality of the genes for which markers are listed in Tables 1-5; and comparing said expression to a control, where said control is representative of the expression of said plurality of genes in a breast cancer sample of said subtype of cancer, and on the basis of said comparison, diagnosing the individual as having said subtype of breast cancer. In a specific embodiment, said subtype of cancer is selected from the group consisting of $ER^-$, *BRCA1* type; $ER^-$, sporadic type; ER+, ER/AGE high type; ER+, ER/AGE low, LN+ type; and ER/AGE low, $LN^-$ type. In another specific embodiment, said control is the average level of expression of a plurality of the genes

for which markers are listed in Table 1, Table 2, Table 3, Table 4 or Table 5. In another specific example, said comparing comprises determining the similarity of the expression of the genes for which markers are listed in each of Tables 1-5 in said sample taken from said individual to a control level of expression of the same genes for each of Tables 1-5, and determining whether the level of expression of said genes in said sample is most similar to said control expression of the genes for which markers are listed in Table 1, Table 2, Table 3, Table 4 or Table 5.

[0083] In another embodiment, the invention provides a method of classifying an individual as having a good prognosis or a poor prognosis, comprising: (a) classifying said individual as $ER^-$, *BRCA1*; $ER^-$, sporadic; ER+, ER/AGE high; ER+, ER/AGE low, LN+; or ER+, ER/AGE low, $LN^-$; (b) determining the level of expression of a first plurality of genes in a cell sample taken from the individual relative to a control, said first plurality of genes comprising two of the genes corresponding to the markers Table 1 if said individual is classified as $ER^-$, *BRCA1*; Table 2 if said individual is classified as $ER^-$, sporadic; Table 3 if said individual is classified as ER+, ER/AGE high; Table 4 if said individual is classified as ER+, ER/AGE low, LN+; or Table 5 if said individual is classified as ER+, ER/AGE low, $LN^-$, wherein said individual is "ER/AGE high" if the ratio of ER expression to age exceeds a predetermined value, and "ER/AGE low" if the ratio of ER expression to age does not exceed said predetermined value. In a specific embodiment of this method, said predetermined value of ER calculated as ER = 0.1(AGE - 42.5), wherein AGE is the age of said individual. In another specific embodiment, said individual is $ER^-$, *BRCA1*, and said plurality of genes comprises (*i.e.*, contains at least) 1, 2, 3, 4, 5, 10 or all of the genes for which markers are listed in Table 1. In another specific embodiment, said individual is $ER^-$, sporadic, and said plurality of genes comprises (*i.e.*, contains at least) 1, 2, 3, 4, 5, 10 or all of the genes for which markers are listed in Table 2. In another specific embodiment, said individual is ER+, ER/AGE high, and said plurality of genes comprises (*i.e.*, contains at least) 1, 2, 3, 4, 5, 10 or all of the genes for which markers are listed in Table 3. In another specific embodiment, said individual is ER+, ER/AGE low, LN+, and said plurality of genes comprises (*i.e.*, contains at least) 1, 2, 3, 4, 5, 10 or all of the genes for which markers are listed in Table 4. In another specific embodiment, said individual is ER+, ER/AGE low, $LN^-$, and said plurality of genes comprises (*i.e.*, contains at least) 1, 2, 3, 4, 5, 10 or all of the genes for which markers are listed in Table 5. In another specific embodiment, the method additionally comprises determining in said cell sample the level of expression, relative to a

control, of a second plurality of genes for which markers are not found in Tables 1-5, wherein said second plurality of genes is informative for prognosis.

[0084] Where information is available regarding the LN status of a breast cancer patient, the patient may identified as having a "very good prognosis," an "intermediate prognosis," or a poor prognosis, which enables the refinement of treatment. In one embodiment, the invention provides a method of assigning a therapeutic regimen to a breast cancer patient, comprising: (a) classifying said patient as having a "poor prognosis," "intermediate prognosis," or "very good prognosis" on the basis of the levels of expression of at least five genes for which markers are listed in Table 1, Table 2, Table 3, Table 4 or Table 5; and (b) assigning said patient a therapeutic regimen, said therapeutic regimen (i) comprising no adjuvant chemotherapy if the patient is lymph node negative and is classified as having a good prognosis or an intermediate prognosis, or (ii) comprising chemotherapy if said patient has any other combination of lymph node status and expression profile.

[0085] In another embodiment, a breast cancer patient is assigned a prognosis by a method comprising (a) determining the breast cancer patient's age, ER status, LN status and tumor type; (b) classifying said patient as $ER^-$, sporadic; $ER^-$, *BRCA1*; ER+, ER/AGE high; ER+, ER/AGE low, LN+; or ER+, ER/AGE low, $LN^-$; (c) determining the level of expression of at least five genes in a cell sample taken from said breast cancer patient wherein markers for said at least five genes are listed in Table 1 if said patient is classified as $ER^-$, sporadic; Table 2 if said patient is classified as $ER^-$, *BRCA1*; Table 3 if said patient is classified as ER+, ER/AGE high; Table 4 if said patient is classified as ER+, ER/AGE low, LN+; or Table 5 if said patient is classified as ER+, ER/AGE high, $LN^-$; (d) determining the similarity of the level of expression of said at least five genes to control levels of expression of said at least five genes to obtain a patient similarity value; (e) comparing said patient similarity value to selected first and second threshold values of similarity of said level of expression of said genes to said control levels of expression to obtain first and second similarity threshold values, respectively, wherein said second similarity threshold indicates greater similarity to said control levels of expression than does said first similarity threshold; and (f) classifying said breast cancer patient as having a first prognosis if said patient similarity value exceeds said first and said second threshold similarity values, a second prognosis if said patient similarity value exceeds said first threshold similarity value but does not exceed said second threshold similarity value, and a third prognosis if said patient similarity value does not exceed said first threshold similarity value or said second threshold similarity value. In a specific embodiment of the method, said first prognosis is a "very good prognosis," said

second prognosis is an "intermediate prognosis," and said third prognosis is a "poor prognosis," wherein said breast cancer patient is assigned a therapeutic regimen comprising no adjuvant chemotherapy if the patient is lymph node negative and is classified as having a good prognosis or an intermediate prognosis, or comprising chemotherapy if said patient has any other combination of lymph node status and expression profile.

[0086] The invention also provides a method of assigning a therapeutic regimen to a breast cancer patient, comprising: (a) determining the lymph node status for said patient; (b) determining the level of expression of at least five genes for which markers are listed in Table 5 in a cell sample from said patient, thereby generating an expression profile; (c) classifying said patient as having a "poor prognosis," "intermediate prognosis," or "very good prognosis" on the basis of said expression profile; and (d) assigning said patient a therapeutic regimen, said therapeutic regimen comprising no adjuvant chemotherapy if the patient is lymph node negative and is classified as having a good prognosis or an intermediate prognosis, or comprising chemotherapy if said patient has any other combination of lymph node status and classification. In a specific embodiment of this method, said therapeutic regimen assigned to lymph node negative patients classified as having an "intermediate prognosis" additionally comprises adjuvant hormonal therapy. In another specific embodiment of this method, said classifying step (c) is carried out by a method comprising: (a) rank ordering in descending order a plurality of breast cancer tumor samples that compose a pool of breast cancer tumor samples by the degree of similarity between the level of expression of said at least five genes in each of said tumor samples and the level of expression of said at least five genes across all remaining tumor samples that compose said pool, said degree of similarity being expressed as a similarity value; (b) determining an acceptable number of false negatives in said classifying step, wherein a false negative is a breast cancer patient for whom the expression levels of said at least five genes in said cell sample predicts that said breast cancer patient will have no distant metastases within the first five years after initial diagnosis, but who has had a distant metastasis within the first five years after initial diagnosis; (c) determining a similarity value above which in said rank ordered list said acceptable number of tumor samples or fewer are false negatives; (d) selecting said similarity value determined in step (c) as a first threshold similarity value; (e) selecting a second similarity value, greater than said first similarity value, as a second threshold similarity value; and (f) determining the similarity between the level of expression of each of said at least five genes in a breast cancer tumor sample from the breast cancer patient and the level of expression of each of said respective at least five genes in said pool, to obtain a patient similarity value, wherein if said patient similarity value

equals or exceeds said second threshold similarity value, said patient is classified as having a "very good prognosis"; if said patient similarity value equals or exceeds said first threshold similarity value, but is less than said second threshold similarity value, said patient is classified as having an "intermediate prognosis"; and if said patient similarity value is less than said first threshold similarity value, said patient is classified as having a "poor prognosis." Another specific embodiment of this method comprises determining the estrogen receptor (ER) status of said patient, wherein if said patient is ER positive and lymph node negative, said therapeutic regimen assigned to said patient additionally comprises adjuvant hormonal therapy.

### 5.4.3 IMPROVING SENSITIVITY TO EXPRESSION LEVEL DIFFERENCES

[0087] In using the markers disclosed herein, and, indeed, using any sets of markers to differentiate an individual having one phenotype from another individual having a second phenotype, one can compare the absolute expression of each of the markers in a sample to a control; for example, the control can be the average level of expression of each of the markers, respectively, in a pool of individuals. To increase the sensitivity of the comparison, however, the expression level values are preferably transformed in a number of ways.

[0088] For example, the expression level of each of the markers can be normalized by the average expression level of all markers the expression level of which is determined, or by the average expression level of a set of control genes. Thus, in one embodiment, the markers are represented by probes on a microarray, and the expression level of each of the markers is normalized by the mean or median expression level across all of the genes represented on the microarray, including any non-marker genes. In a specific embodiment, the normalization is carried out by dividing the median or mean level of expression of all of the genes on the microarray. In another embodiment, the expression levels of the markers is normalized by the mean or median level of expression of a set of control markers. In a specific embodiment, the control markers comprise a set of housekeeping genes. In another specific embodiment, the normalization is accomplished by dividing by the median or mean expression level of the control genes.

[0089] The sensitivity of a marker-based assay will also be increased if the expression levels of individual markers are compared to the expression of the same markers in a pool of samples. Preferably, the comparison is to the mean or median expression level of each the marker genes in the pool of samples. Such a comparison may be accomplished, for example, by dividing by the mean or median expression level of the pool for each of the markers from

the expression level each of the markers in the sample. This has the effect of accentuating the relative differences in expression between markers in the sample and markers in the pool as a whole, making comparisons more sensitive and more likely to produce meaningful results that the use of absolute expression levels alone. The expression level data may be transformed in any convenient way; preferably, the expression level data for all is log transformed before means or medians are taken.

[0090] In performing comparisons to a pool, two approaches may be used. First, the expression levels of the markers in the sample may be compared to the expression level of those markers in the pool, where nucleic acid derived from the sample and nucleic acid derived from the pool are hybridized during the course of a single experiment. Such an approach requires that new pool nucleic acid be generated for each comparison or limited numbers of comparisons, and is therefore limited by the amount of nucleic acid available. Alternatively, and preferably, the expression levels in a pool, whether normalized and/or transformed or not, are stored on a computer, or on computer-readable media, to be used in comparisons to the individual expression level data from the sample (*i.e.*, single-channel data).

[0091] Thus, the current invention provides the following method of classifying a first cell or organism as having one of at least two different phenotypes, where the different phenotypes comprise a first phenotype and a second phenotype. The level of expression of each of a plurality of markers in a first sample from the first cell or organism is compared to the level of expression of each of said markers, respectively, in a pooled sample from a plurality of cells or organisms, the plurality of cells or organisms comprising different cells or organisms exhibiting said at least two different phenotypes, respectively, to produce a first compared value. The first compared value is then compared to a second compared value, wherein said second compared value is the product of a method comprising comparing the level of expression of each of said markers in a sample from a cell or organism characterized as having said first phenotype to the level of expression of each of said markers, respectively, in the pooled sample. The first compared value is then compared to a third compared value, wherein said third compared value is the product of a method comprising comparing the level of expression of each of the markers in a sample from a cell or organism characterized as having the second phenotype to the level of expression of each of the markers, respectively, in the pooled sample. In specific embodiments, the marker can be a gene, a protein encoded by the gene, etc. Optionally, the first compared value can be compared to additional compared values, respectively, where each additional compared value is the product of a

method comprising comparing the level of expression of each of said markers in a sample from a cell or organism characterized as having a phenotype different from said first and second phenotypes but included among the at least two different phenotypes, to the level of expression of each of said genes, respectively, in said pooled sample. Finally, a determination is made as to which of said second, third, and, if present, one or more additional compared values, said first compared value is most similar, wherein the first cell or organism is determined to have the phenotype of the cell or organism used to produce said compared value most similar to said first compared value.

[0092] In a specific embodiment of this method, the compared values are each ratios of the levels of expression of each of said genes. In another specific embodiment, each of the levels of expression of each of the genes in the pooled sample are normalized prior to any of the comparing steps. In a more specific embodiment, the normalization of the levels of expression is carried out by dividing by the median or mean level of the expression of each of the genes or dividing by the mean or median level of expression of one or more housekeeping genes in the pooled sample from said cell or organism. In another specific embodiment, the normalized levels of expression are subjected to a log transform, and the comparing steps comprise subtracting the log transform from the log of the levels of expression of each of the genes in the sample. In another specific embodiment, the two or more different phenotypes are different stages of a disease or disorder. In still another specific embodiment, the two or more different phenotypes are different prognoses of a disease or disorder. In yet another specific embodiment, the levels of expression of each of the genes, respectively, in the pooled sample or said levels of expression of each of said genes in a sample from the cell or organism characterized as having the first phenotype, second phenotype, or said phenotype different from said first and second phenotypes, respectively, are stored on a computer or on a computer-readable medium.

[0093] In another specific embodiment, the two phenotypes are good prognosis and poor prognosis. In a more specific embodiment, the two phenotypes are good prognosis and poor prognosis for an individual that is identified as having $ER^-$, *BRCA1* status, $ER^-$, sporadic status, ER+, ER/AGE high status, ER+, ER/AGE low, LN+ status, or ER+, ER/AGE low, LN+ status.

[0094] In another specific embodiment, the comparison is made between the expression of each of the genes in the sample and the expression of the same genes in a pool representing only one of two or more phenotypes. In the context of prognosis-correlated genes, for example, one can compare the expression levels of prognosis-related genes in a sample to the

average level of the expression of the same genes in a "good prognosis" pool of samples (as opposed to a pool of samples that include samples from patients having poor prognoses and good prognoses). Thus, in this method, a sample is classified as having a good prognosis if the level of expression of prognosis-correlated genes exceeds a chosen coefficient of correlation to the average "good prognosis" expression profile (*i.e.*, the level of expression of prognosis-correlated genes in a pool of samples from patients having a "good prognosis." Patients whose expression levels correlate more poorly with the "good prognosis" expression profile (*i.e.*, whose correlation coefficient fails to exceed the chosen coefficient) are classified as having a poor prognosis.

[0095] Where individuals are classified on the basis of phenotypic, genotypic, or clinical characteristics into patient subsets, the pool of samples may be a pool of samples for the phenotype that includes samples representing each of the patient subsets. Alternatively, the pool of samples may be a pool of samples for the phenotype representing only the specific patient subset. For example, where an individual is classified as ER+, sporadic, the pool of samples to which the individual's sample is compared may be a pool of samples from ER+, sporadic individuals having a good prognosis only, or may be a pool of samples of individuals having a good prognosis, without regard to ER status or mutation type.

[0096] The method can be applied to a plurality of patient subsets. For example, in a specific embodiment, the phenotype is good prognosis, and the individual is classified into one of the following patient subsets: $ER^-$, *BRCA1* status, $ER^-$, sporadic status, ER+, ER/AGE high status, ER+, ER/AGE low, LN+ status, or ER+, ER/AGE low, LN+ status. A set of markers informative for prognosis for the patient subset into which the individual is classified is then used to determine the likely prognosis for the individual. A sample is classified as coming from an individual having a good prognosis if the level of expression of prognosis-correlated genes for the particular subset into which the individual is classified exceeds a chosen coefficient of correlation to the average "good prognosis" expression profile (*i.e.*, the level of expression of prognosis-correlated genes in a pool of samples from patients within the subclass having a "good prognosis"). Patients whose expression levels correlate more poorly with the "good prognosis" expression profile (*i.e.*, whose correlation coefficient fails to exceed the chosen coefficient) are classified as having a poor prognosis.

[0097] Of course, single-channel data may also be used without specific comparison to a mathematical sample pool. For example, a sample may be classified as having a first or a second phenotype, wherein the first and second phenotypes are related, by calculating the similarity between the expression of at least 5 markers in the sample, where the markers are

correlated with the first or second phenotype, to the expression of the same markers in a first phenotype template and a second phenotype template, by (a) labeling nucleic acids derived from a sample with a fluorophore to obtain a pool of fluorophore-labeled nucleic acids; (b) contacting said fluorophore-labeled nucleic acid with a microarray under conditions such that hybridization can occur, detecting at each of a plurality of discrete loci on the microarray a fluorescent emission signal from said fluorophore-labeled nucleic acid that is bound to said microarray under said conditions; and (c) determining the similarity of marker gene expression in the individual sample to the first and second templates, wherein if said expression is more similar to the first template, the sample is classified as having the first phenotype, and if said expression is more similar to the second template, the sample is classified as having the second phenotype.

[0098] In a specific embodiment of the above method, the first phenotype is a good prognosis of breast cancer, the sample is a sample from an individual that has been classified into a patient subset, and the first and second templates are templates for the phenotype for the particular patient subset. In a more specific embodiment, for example, the first phenotype is a good prognosis, the second phenotype is a poor prognosis, the patient is classified into an $ER^-$, sporadic patient subset, an $ER^-$, *BRCA1* subset, an ER+, ER/AGE high subset, an ER+, ER/AGE low, LN+ subset, or an ER+, ER/AGE low, LN+ subset, and said first and second templates are templates derived from the expression of the marker genes in individuals having a good prognosis and a poor prognosis, respectively, wherein said individuals are all of the patient subset into which said patient is classified.

## 5.5 DETERMINATION OF MARKER GENE EXPRESSION LEVELS

### 5.5.1 METHODS

[0099] The expression levels of the marker genes in a sample may be determined by any means known in the art. The expression level may be determined by isolating and determining the level (*i.e.*, amount) of nucleic acid transcribed from each marker gene. Alternatively, or additionally, the level of specific proteins encoded by a marker gene may be determined.

[00100] The level of expression of specific marker genes can be accomplished by determining the amount of mRNA, or polynucleotides derived therefrom, present in a sample. Any method for determining RNA levels can be used. For example, RNA is isolated from a sample and separated on an agarose gel. The separated RNA is then transferred to a solid support, such as a filter. Nucleic acid probes representing one or more markers are then

hybridized to the filter by northern hybridization, and the amount of marker-derived RNA is determined. Such determination can be visual, or machine-aided, for example, by use of a densitometer. Another method of determining RNA levels is by use of a dot-blot or a slot-blot. In this method, RNA, or nucleic acid derived therefrom, from a sample is labeled. The RNA or nucleic acid derived therefrom is then hybridized to a filter containing oligonucleotides derived from one or more marker genes, wherein the oligonucleotides are placed upon the filter at discrete, easily-identifiable locations. Hybridization, or lack thereof, of the labeled RNA to the filter-bound oligonucleotides is determined visually or by densitometer. Polynucleotides can be labeled using a radiolabel or a fluorescent (*i.e.*, visible) label.

[00101] These examples are not intended to be limiting; other methods of determining RNA abundance are known in the art.

[00102] The level of expression of particular marker genes may also be assessed by determining the level of the specific protein expressed from the marker genes. This can be accomplished, for example, by separation of proteins from a sample on a polyacrylamide gel, followed by identification of specific marker-derived proteins using antibodies in a western blot. Alternatively, proteins can be separated by two-dimensional gel electrophoresis systems. Two-dimensional gel electrophoresis is well-known in the art and typically involves isoelectric focusing along a first dimension followed by SDS-PAGE electrophoresis along a second dimension. *See, e.g.*, Hames *et al*, 1990, GEL ELECTROPHORESIS OF PROTEINS: A PRACTICAL APPROACH, IRL Press, New York; Shevchenko *et al.*, *Proc. Nat'l Acad. Sci.* USA 93:1440-1445 (1996); Sagliocco *et al.*, *Yeast* 12:1519-1533 (1996); Lander, *Science* 274:536-539 (1996). The resulting electropherograms can be analyzed by numerous techniques, including mass spectrometric techniques, western blotting and immunoblot analysis using polyclonal and monoclonal antibodies.

[00103] Alternatively, marker-derived protein levels can be determined by constructing an antibody microarray in which binding sites comprise immobilized, preferably monoclonal, antibodies specific to a plurality of protein species encoded by the cell genome. Preferably, antibodies are present for a substantial fraction of the marker-derived proteins of interest. Methods for making monoclonal antibodies are well known (*see, e.g.*, Harlow and Lane, 1988, ANTIBODIES: A LABORATORY MANUAL, Cold Spring Harbor, New York, which is incorporated in its entirety for all purposes). In one embodiment, monoclonal antibodies are raised against synthetic peptide fragments designed based on genomic sequence of the cell. With such an antibody array, proteins from the cell are contacted to the array, and their

binding is assayed with assays known in the art. Generally, the expression, and the level of expression, of proteins of diagnostic or prognostic interest can be detected through immunohistochemical staining of tissue slices or sections.

[00104] Finally, expression of marker genes in a number of tissue specimens may be characterized using a "tissue array" (Kononen *et al.*, *Nat. Med* 4(7):844-7 (1998)). In a tissue array, multiple tissue samples are assessed on the same microarray. The arrays allow *in situ* detection of RNA and protein levels; consecutive sections allow the analysis of multiple samples simultaneously.

### 5.5.2 MICROARRAYS

[00105] In preferred embodiments, polynucleotide microarrays are used to measure expression so that the expression status of each of the markers above is assessed simultaneously. In a specific embodiment, the invention provides for oligonucleotide or cDNA arrays comprising probes hybridizable to the genes corresponding to each of the marker sets described above (*i.e.*, markers informative for $ER^-$, sporadic individuals, markers informative for $ER^-$, *BRCA1* individuals, markers informative for ER+, ER/AGE high individuals, markers informative for ER+, ER/AGE low, LN+ individuals, and markers informative for ER+, ER/AGE low, $LN^-$ individuals, as shown in Tables 1-5). Any of the microarrays described herein may be provided in a sealed container in a kit.

[00106] The invention provides microarrays containing probes useful for the prognosis of any breast cancer patient, or for breast cancer patients classified into one of a plurality of patient subsets. In particular, the invention provides polynucleotide arrays comprising probes to a subset or subsets of at least 5, 10, 15, 20, 25 or more of the genetic markers, or up to the full set of markers, in any of Tables 1-5, which distinguish between patients with good and poor prognosis. In certain embodiments, therefore, the invention provides microarrays comprising probes for a plurality of the genes for which markers are listed in Tables 1, 2, 3, 4 or 5. In a specific embodiment, the microarray of the invention comprises 1, 2, 3, 4, 5 or 10 of the markers in Table 1, at least five of the markers in Table 2; 1, 2, 3, 4, 5 or 10 of the markers in Table 3; 1, 2, 3, 4, 5 or 10 of the markers in Table 4; or 1, 2, 3, 4, 5 or 10 of the markers in Table 1. In other embodiments, the microarray comprises probes for 1, 2, 3, 4, 5, or 10 of the markers shown in any two, three or four of Tables 1-5, or all of Tables 1-5. In other embodiments, the microarray of the invention contains each of the markers in Table 1, Table 2, Table 3, Table 4, or Table 5. In another embodiment, the microarray contains all of the markers shown in Tables 1-5. In specific embodiments, the array comprises probes

derived only from the markers listed in Table 1, Table 2, Table 3, Table 4, or Table 5; probes derived from any two of Tables 1-5; any three of Tables 1-5; any four of Tables 1-5; or all of Tables 1-5.

[00107] In other embodiments, the array comprises a plurality of probes derived from markers listed in any of Tables 1-5 in combination with a plurality of other probes, derived from markers not listed in any of Tables 1-5, that are identified as informative for the prognosis of breast cancer.

[00108] In specific embodiments, the invention provides polynucleotide arrays in which the breast cancer prognosis markers described herein in Tables 1, 2, 3, 4 and/or 5 comprise at least 50%, 60%, 70%, 80%, 85%, 90%, 95% or 98% of the probes on said array. In another specific embodiment, the microarray comprises a plurality of probes, wherein said plurality of probes comprise probes complementary and hybridizable to 75% of the genes for which markers are listed in Table 1; probes complementary and hybridizable to 75% of the genes for which markers are listed in Table 2; probes complementary and hybridizable to 75% of the genes for which markers are listed in Table 3; probes complementary and hybridizable to 75% of the genes for which markers are listed in Table 4; and probes complementary and hybridizable to 75% of the genes for which markers are listed in Table 5, wherein said probes, in total, comprise 50% of the probes on said microarray.

[00109] In yet another specific embodiment, microarrays that are used in the methods disclosed herein optionally comprise markers additional to at least some of the markers listed in Tables 1-5. For example, in a specific embodiment, the microarray is a screening or scanning array as described in Altschuler *et al.*, International Publication WO 02/18646, published March 7, 2002 and Scherer *et al.*, International Publication WO 02/16650, published February 28, 2002. The scanning and screening arrays comprise regularly-spaced, positionally-addressable probes derived from genomic nucleic acid sequence, both expressed and unexpressed. Such arrays may comprise probes corresponding to a subset of, or all of, the markers listed in Tables 1-5, or a subset thereof as described above, and can be used to monitor marker expression in the same way as a microarray containing only markers listed in Tables 1-5.

[00110] In yet another specific embodiment, the microarray is a commercially-available cDNA microarray that comprises at least five of the markers listed in Tables 1-5. Preferably, a commercially-available cDNA microarray comprises all of the markers listed in Tables 1-5. However, such a microarray may comprise 5, 10, 15, 25 or more of the markers in any of Tables 1-5, up to the maximum number of markers in a Table, and may comprise all

of the markers in any one of Tables 1-5, and a subset of another of Tables 1-5, or subsets of each as described above. In a specific embodiment of the microarrays used in the methods disclosed herein, the markers that are all or a portion of Tables 1-5 make up at least 50%, 60%, 70%, 80%, 90%, 95% or 98% of the probes on the microarray.

[00111] General methods pertaining to the construction of microarrays comprising the marker sets and/or subsets above are described in the following sections.

5.5.2.1 CONSTRUCTION OF MICROARRAYS

[00112] Microarrays are prepared by selecting probes which comprise a polynucleotide sequence, and then immobilizing such probes to a solid support or surface. For example, the probes may comprise DNA sequences, RNA sequences, or copolymer sequences of DNA and RNA. The polynucleotide sequences of the probes may also comprise DNA and/or RNA analogues, or combinations thereof. For example, the polynucleotide sequences of the probes may be full or partial fragments of genomic DNA. The polynucleotide sequences of the probes may also be synthesized nucleotide sequences, such as synthetic oligonucleotide sequences. The probe sequences can be synthesized either enzymatically *in vivo*, enzymatically *in vitro* (*e.g.*, by PCR), or non-enzymatically *in vitro*.

[00113] The probe or probes used in the methods of the invention are preferably immobilized to a solid support which may be either porous or non-porous. For example, the probes of the invention may be polynucleotide sequences which are attached to a nitrocellulose or nylon membrane or filter covalently at either the 3' or the 5' end of the polynucleotide. Such hybridization probes are well known in the art (see, *e.g.*, Sambrook *et al.*, MOLECULAR CLONING - A LABORATORY MANUAL (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989). Alternatively, the solid support or surface may be a glass or plastic surface. In a particularly preferred embodiment, hybridization levels are measured to microarrays of probes consisting of a solid phase on the surface of which are immobilized a population of polynucleotides, such as a population of DNA or DNA mimics, or, alternatively, a population of RNA or RNA mimics. The solid phase may be a nonporous or, optionally, a porous material such as a gel.

[0100] In preferred embodiments, a microarray comprises a support or surface with an ordered array of binding (*e.g.*, hybridization) sites or "probes" each representing one of the markers described herein. Preferably the microarrays are addressable arrays, and more preferably positionally addressable arrays. More specifically, each probe of the array is preferably located at a known, predetermined position on the solid support such that the

identity (*i.e.*, the sequence) of each probe can be determined from its position in the array (*i.e.*, on the support or surface). In preferred embodiments, each probe is covalently attached to the solid support at a single site.

[0101] Microarrays can be made in a number of ways, of which several are described below. However produced, microarrays share certain characteristics. The arrays are reproducible, allowing multiple copies of a given array to be produced and easily compared with each other. Preferably, microarrays are made from materials that are stable under binding (*e.g.*, nucleic acid hybridization) conditions. The microarrays are preferably small, e.g., between 1 $cm^2$ and 25 $cm^2$, between 12 $cm^2$ and 13 $cm^2$, or 3 $cm^2$. However, larger arrays are also contemplated and may be preferable, e.g., for use in screening arrays. Preferably, a given binding site or unique set of binding sites in the microarray will specifically bind (*e.g.*, hybridize) to the product of a single gene in a cell (*e.g.*, to a specific mRNA, or to a specific cDNA derived therefrom). However, in general, other related or similar sequences will cross hybridize to a given binding site.

[0102] The microarrays of the present invention include one or more test probes, each of which has a polynucleotide sequence that is complementary to a subsequence of RNA or DNA to be detected. Preferably, the position of each probe on the solid surface is known. Indeed, the microarrays are preferably positionally addressable arrays. Specifically, each probe of the array is preferably located at a known, predetermined position on the solid support such that the identity (*i.e.*, the sequence) of each probe can be determined from its position on the array (*i.e.*, on the support or surface).

[0103] According to the invention, the microarray is an array (*i.e.*, a matrix) in which each position represents one of the markers described herein. For example, each position can contain a DNA or DNA analogue based on genomic DNA to which a particular RNA or cDNA transcribed from that genetic marker can specifically hybridize. The DNA or DNA analogue can be, *e.g.*, a synthetic oligomer or a gene fragment. In one embodiment, probes representing each of the markers is present on the array. In a preferred embodiment, the array comprises probes for each of the markers listed in Tables 1-5.

### 5.5.2.2 PREPARING PROBES FOR MICROARRAYS

[0104] As noted above, the "probe" to which a particular polynucleotide molecule specifically hybridizes according to the invention contains a complementary genomic polynucleotide sequence. The probes of the microarray preferably consist of nucleotide sequences of no more than 1,000 nucleotides. In some embodiments, the probes of the array

consist of nucleotide sequences of 10 to 1,000 nucleotides. In a preferred embodiment, the nucleotide sequences of the probes are in the range of 10-200 nucleotides in length and are genomic sequences of a species of organism, such that a plurality of different probes is present, with sequences complementary and thus capable of hybridizing to the genome of such a species of organism, sequentially tiled across all or a portion of such genome. In other specific embodiments, the probes are in the range of 10-30 nucleotides in length, in the range of 10-40 nucleotides in length, in the range of 20-50 nucleotides in length, in the range of 40-80 nucleotides in length, in the range of 50-150 nucleotides in length, in the range of 80-120 nucleotides in length, and most preferably are 60 nucleotides in length.

[0105] The probes may comprise DNA or DNA "mimics" (*e.g.*, derivatives and analogues) corresponding to a portion of an organism's genome. In another embodiment, the probes of the microarray are complementary RNA or RNA mimics. DNA mimics are polymers composed of subunits capable of specific, Watson-Crick-like hybridization with DNA, or of specific hybridization with RNA. The nucleic acids can be modified at the base moiety, at the sugar moiety, or at the phosphate backbone. Exemplary DNA mimics include, *e.g.*, phosphorothioates.

[0106] DNA can be obtained, *e.g.*, by polymerase chain reaction (PCR) amplification of genomic DNA or cloned sequences. PCR primers are preferably chosen based on a known sequence of the genome that will result in amplification of specific fragments of genomic DNA. Computer programs that are well known in the art are useful in the design of primers with the required specificity and optimal amplification properties, such as *Oligo* version 5.0 (National Biosciences). Typically each probe on the microarray will be between 10 bases and 50,000 bases, usually between 300 bases and 1,000 bases in length. PCR methods are well known in the art, and are described, for example, in Innis *et al.*, eds., PCR PROTOCOLS: A GUIDE TO METHODS AND APPLICATIONS, Academic Press Inc., San Diego, CA (1990). It will be apparent to one skilled in the art that controlled robotic systems are useful for isolating and amplifying nucleic acids.

[0107] An alternative, preferred means for generating the polynucleotide probes of the microarray is by synthesis of synthetic polynucleotides or oligonucleotides, *e.g.*, using N-phosphonate or phosphoramidite chemistries (Froehler *et al.*, *Nucleic Acid Res.* 14:5399-5407 (1986); McBride *et al.*, *Tetrahedron Lett.* 24:246-248 (1983)). Synthetic sequences are typically between about 10 and about 500 bases in length, more typically between about 20 and about 100 bases, and most preferably between about 40 and about 70 bases in length. In some embodiments, synthetic nucleic acids include non-natural bases, such as, but by no

means limited to, inosine. As noted above, nucleic acid analogues may be used as binding sites for hybridization. An example of a suitable nucleic acid analogue is peptide nucleic acid (*see*, *e.g.*, Egholm *et al.*, *Nature* 363:566-568 (1993); U.S. Patent No. 5,539,083).

[0108] Probes are preferably selected using an algorithm that takes into account binding energies, base composition, sequence complexity, cross-hybridization binding energies, and secondary structure. *See* Friend *et al.*, International Patent Publication WO 01/05935, published January 25, 2001; Hughes *et al.*, *Nat. Biotech.* 19:342-7 (2001).

[0109] A skilled artisan will also appreciate that positive control probes, *e.g.*, probes known to be complementary and hybridizable to sequences in the target polynucleotide molecules, and negative control probes, *e.g.*, probes known to not be complementary and hybridizable to sequences in the target polynucleotide molecules, should be included on the array. In one embodiment, positive controls are synthesized along the perimeter of the array. In another embodiment, positive controls are synthesized in diagonal stripes across the array. In still another embodiment, the reverse complement for each probe is synthesized next to the position of the probe to serve as a negative control. In yet another embodiment, sequences from other species of organism are used as negative controls or as "spike-in" controls.

### 5.5.2.3 ATTACHING PROBES TO THE SOLID SURFACE

[0110] The probes are attached to a solid support or surface, which may be made, *e.g.*, from glass, plastic (*e.g.*, polypropylene, nylon), polyacrylamide, nitrocellulose, gel, or other porous or nonporous material. A preferred method for attaching the nucleic acids to a surface is by printing on glass plates, as is described generally by Schena *et al*, *Science* 270:467-470 (1995). This method is especially useful for preparing microarrays of cDNA (See also, DeRisi *et al*, *Nature Genetics* 14:457-460 (1996); Shalon *et al.*, *Genome Res. 6* :639-645 (1996); and Schena *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 93:10539-11286 (1995)).

[0111] A second preferred method for making microarrays is by making high-density oligonucleotide arrays. Techniques are known for producing arrays containing thousands of oligonucleotides complementary to defined sequences, at defined locations on a surface using photolithographic techniques for synthesis *in situ* (see, Fodor *et al.*, 1991, *Science* 251:767-773; Pease *et al.*, 1994, *Proc. Natl. Acad. Sci. U.S.A.* 91:5022-5026; Lockhart *et al.*, 1996, *Nature Biotechnology* 14:1675; U.S. Patent Nos. 5,578,832; 5,556,752; and 5,510,270) or other methods for rapid synthesis and deposition of defined oligonucleotides (Blanchard *et al.*, *Biosensors & Bioelectronics* 11:687-690). When these methods are used, oligonucleotides (*e.g.*, 60-mers) of known sequence are synthesized directly on a surface such

as a derivatized glass slide. Usually, the array produced is redundant, with several oligonucleotide molecules per RNA.

[0112] Other methods for making microarrays, *e.g.*, by masking (Maskos and Southern, 1992, *Nuc. Acids. Res.* 20:1679-1684), may also be used. In principle, and as noted *supra*, any type of array, for example, dot blots on a nylon hybridization membrane (see Sambrook *et al.*, MOLECULAR CLONING - A LABORATORY MANUAL (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989)) could be used. However, as will be recognized by those skilled in the art, very small arrays will frequently be preferred because hybridization volumes will be smaller.

[0113] In one embodiment, the arrays of the present invention are prepared by synthesizing polynucleotide probes on a support. In such an embodiment, polynucleotide probes are attached to the support covalently at either the 3′ or the 5′ end of the polynucleotide.

[0114] In a particularly preferred embodiment, microarrays of the invention are manufactured by means of an ink jet printing device for oligonucleotide synthesis, *e.g.*, using the methods and systems described by Blanchard in U.S. Pat. No. 6,028,189; Blanchard et al., 1996, *Biosensors and Bioelectronics* 11:687-690; Blanchard, 1998, in Synthetic DNA Arrays in Genetic Engineering, Vol. 20, J.K. Setlow, Ed., Plenum Press, New York at pages 111-123. Specifically, the oligonucleotide probes in such microarrays are preferably synthesized in arrays, e.g., on a glass slide, by serially depositing individual nucleotide bases in "microdroplets" of a high surface tension solvent such as propylene carbonate. The microdroplets have small volumes (e.g., 100 pL or less, more preferably 50 pL or less) and are separated from each other on the microarray (e.g., by hydrophobic domains) to form circular surface tension wells which define the locations of the array elements (i.e., the different probes). Microarrays manufactured by this ink-jet method are typically of high density, preferably having a density of at least about 2,500 different probes per 1 $cm^2$. The polynucleotide probes are attached to the support covalently at either the 3′ or the 5′ end of the polynucleotide.

### 5.5.2.4 TARGET POLYNUCLEOTIDE MOLECULES

[0115] The polynucleotide molecules which may be analyzed by the present invention (the "target polynucleotide molecules") may be from any clinically relevant source, but are expressed RNA or a nucleic acid derived therefrom (*e.g.*, cDNA or amplified RNA derived from cDNA that incorporates an RNA polymerase promoter), including naturally occurring nucleic acid molecules, as well as synthetic nucleic acid molecules. In one embodiment, the

target polynucleotide molecules comprise RNA, including, but by no means limited to, total cellular RNA, poly(A)$^+$ messenger RNA (mRNA) or fraction thereof, cytoplasmic mRNA, or RNA transcribed from cDNA (*i.e.*, cRNA; see, *e.g.*, Linsley & Schelter, U.S. Patent Application No. 09/411,074, filed October 4, 1999, or U.S. Patent Nos. 5,545,522, 5,891,636, or 5,716,785). Methods for preparing total and poly(A)$^+$ RNA are well known in the art, and are described generally, *e.g.*, in Sambrook *et al.*, MOLECULAR CLONING - A LABORATORY MANUAL (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989). In one embodiment, RNA is extracted from cells of the various types of interest in this invention using guanidinium thiocyanate lysis followed by CsCl centrifugation (Chirgwin *et al.*, 1979, *Biochemistry* 18:5294-5299). In another embodiment, total RNA is extracted using a silica gel-based column, commercially available examples of which include RNeasy (Qiagen, Valencia, California) and StrataPrep (Stratagene, La Jolla, California). In an alternative embodiment, which is preferred for *S. cerevisiae*, RNA is extracted from cells using phenol and chloroform, as described in Ausubel *et al.*, eds., 1989, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Vol. III, Green Publishing Associates, Inc., John Wiley & Sons, Inc., New York, at pp. 13.12.1-13.12.5). Poly(A)$^+$ RNA can be selected, *e.g.*, by selection with oligo-dT cellulose or, alternatively, by oligo-dT primed reverse transcription of total cellular RNA. In one embodiment, RNA can be fragmented by methods known in the art, *e.g.*, by incubation with $ZnCl_2$, to generate fragments of RNA. In another embodiment, the polynucleotide molecules analyzed by the invention comprise cDNA, or PCR products of amplified RNA or cDNA.

[0116] In one embodiment, total RNA, mRNA, or nucleic acids derived therefrom, is isolated from a sample taken from a person afflicted with breast cancer. Target polynucleotide molecules that are poorly expressed in particular cells may be enriched using normalization techniques (Bonaldo *et al.*, 1996, *Genome Res.* 6:791-806).

[0117] As described above, the target polynucleotides are detectably labeled at one or more nucleotides. Any method known in the art may be used to detectably label the target polynucleotides. Preferably, this labeling incorporates the label uniformly along the length of the RNA, and more preferably, the labeling is carried out at a high degree of efficiency. One embodiment for this labeling uses oligo-dT primed reverse transcription to incorporate the label; however, conventional methods of this method are biased toward generating 3′ end fragments. Thus, in a preferred embodiment, random primers (*e.g.*, 9-mers) are used in reverse transcription to uniformly incorporate labeled nucleotides over the full length of the

target polynucleotides. Alternatively, random primers may be used in conjunction with PCR methods or T7 promoter-based *in vitro* transcription methods in order to amplify the target polynucleotides.

[0118] In a preferred embodiment, the detectable label is a luminescent label. For example, fluorescent labels, bioluminescent labels, chemiluminescent labels, and colorimetric labels may be used in the present invention. In a highly preferred embodiment, the label is a fluorescent label, such as a fluorescein, a phosphor, a rhodamine, or a polymethine dye derivative. Examples of commercially available fluorescent labels include, for example, fluorescent phosphoramidites such as FluorePrime (Amersham Pharmacia, Piscataway, N.J.), Fluoredite (Millipore, Bedford, Mass.), FAM (ABI, Foster City, Calif.), and Cy3 or Cy5 (Amersham Pharmacia, Piscataway, N.J.). In another embodiment, the detectable label is a radiolabeled nucleotide.

[0119] In a further preferred embodiment, target polynucleotide molecules from a patient sample are labeled differentially from target polynucleotide molecules of a standard. The standard can comprise target polynucleotide molecules from normal individuals (*i.e.*, those not afflicted with breast cancer). In a highly preferred embodiment, the standard comprises target polynucleotide molecules pooled from samples from normal individuals or tumor samples from individuals having sporadic-type breast tumors. In another embodiment, the target polynucleotide molecules are derived from the same individual, but are taken at different time points, and thus indicate the efficacy of a treatment by a change in expression of the markers, or lack thereof, during and after the course of treatment (*i.e.*, chemotherapy, radiation therapy or cryotherapy), wherein a change in the expression of the markers from a poor prognosis pattern to a good prognosis pattern indicates that the treatment is efficacious. In this embodiment, different timepoints are differentially labeled.

#### 5.5.2.5 HYBRIDIZATION TO MICROARRAYS

[0120] Nucleic acid hybridization and wash conditions are chosen so that the target polynucleotide molecules specifically bind or specifically hybridize to the complementary polynucleotide sequences of the array, preferably to a specific array site, wherein its complementary DNA is located.

[0121] Arrays containing double-stranded probe DNA situated thereon are preferably subjected to denaturing conditions to render the DNA single-stranded prior to contacting with the target polynucleotide molecules. Arrays containing single-stranded probe DNA (*e.g.*, synthetic oligodeoxyribonucleic acids) may need to be denatured prior to contacting with the

target polynucleotide molecules, *e.g.*, to remove hairpins or dimers which form due to self complementary sequences.

[0122] Optimal hybridization conditions will depend on the length (*e.g.*, oligomer versus polynucleotide greater than 200 bases) and type (*e.g.*, RNA, or DNA) of probe and target nucleic acids. One of skill in the art will appreciate that as the oligonucleotides become shorter, it may become necessary to adjust their length to achieve a relatively uniform melting temperature for satisfactory hybridization results. General parameters for specific (*i.e.*, stringent) hybridization conditions for nucleic acids are described in Sambrook *et al.*, MOLECULAR CLONING - A LABORATORY MANUAL (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989), and in Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, vol. 2, Current Protocols Publishing, New York (1994). Typical hybridization conditions for the cDNA microarrays of Schena *et al.* are hybridization in 5 X SSC plus 0.2% SDS at 65°C for four hours, followed by washes at 25°C in low stringency wash buffer (1 X SSC plus 0.2% SDS), followed by 10 minutes at 25°C in higher stringency wash buffer (0.1 X SSC plus 0.2% SDS) (Schena *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 93:10614 (1993)). Useful hybridization conditions are also provided in, *e.g.*, Tijessen, 1993, HYBRIDIZATION WITH NUCLEIC ACID PROBES, Elsevier Science Publishers B.V.; and Kricka, 1992, NONISOTOPIC DNA PROBE TECHNIQUES , Academic Press, San Diego, CA.

[0123] Particularly preferred hybridization conditions include hybridization at a temperature at or near the mean melting temperature of the probes (*e.g.*, within 51°C, more preferably within 21°C) in 1 M NaCl, 50 mM MES buffer (pH 6.5), 0.5% sodium sarcosine and 30% formamide.

### 5.5.2.6 SIGNAL DETECTION AND DATA ANALYSIS

[0124] When fluorescently labeled probes are used, the fluorescence emissions at each site of a microarray may be, preferably, detected by scanning confocal laser microscopy. In one embodiment, a separate scan, using the appropriate excitation line, is carried out for each of the two fluorophores used. Alternatively, a laser may be used that allows simultaneous specimen illumination at wavelengths specific to the two fluorophores and emissions from the two fluorophores can be analyzed simultaneously (*see* Shalon *et al.*, 1996, "A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization," *Genome Research* 6:639-645, which is incorporated by reference in its entirety for all purposes). In a preferred embodiment, the arrays are scanned with a laser

fluorescent scanner with a computer controlled X-Y stage and a microscope objective. Sequential excitation of the two fluorophores is achieved with a multi-line, mixed gas laser and the emitted light is split by wavelength and detected with two photomultiplier tubes. Fluorescence laser scanning devices are described in Schena *et al.*, *Genome Res.* 6:639-645 (1996), and in other references cited herein. Alternatively, the fiber-optic bundle described by Ferguson *et al.*, *Nature Biotech.* 14:1681-1684 (1996), may be used to monitor mRNA abundance levels at a large number of sites simultaneously.

[0125] Signals are recorded and, in a preferred embodiment, analyzed by computer, *e.g.*, using a 12 or 16 bit analog to digital board. In one embodiment the scanned image is despeckled using a graphics program (*e.g.*, Hijaak Graphics Suite) and then analyzed using an image gridding program that creates a spreadsheet of the average hybridization at each wavelength at each site. If necessary, an experimentally determined correction for "cross talk" (or overlap) between the channels for the two fluors may be made. For any particular hybridization site on the transcript array, a ratio of the emission of the two fluorophores can be calculated. The ratio is independent of the absolute expression level of the cognate gene, but is useful for genes whose expression is significantly modulated in association with the different breast cancer-related condition.

## 5.6 THERAPEUTIC REGIMENS SPECIFIC TO PATIENT SUBSETS

[0126] The benefit of identifying subsets of individuals that have a common condition, followed by identification of sets of genes informative for those particular subsets of individuals, is that such subdivision and identification tends to more accurately identify the subset of genes responsible for, or most closely associated with, a particular form of the condition. For example, breast cancer is a complex condition brought about by several different molecular mechanisms. ER+ individuals, particularly ER+, ER/AGE high individuals, show an increased level of expression of cell cycle-control genes, and the expression of these genes is highly informative for prognosis in this patient subset (*see* Examples). In $ER^-$ individuals, however, the expression of these genes is not informative for prognosis.

[0127] The set of informative markers, therefore, can be used to assign a particular course of therapy to an individual, *e.g.*, an individual having breast cancer, depending upon the condition subset into which the individual is classified. In one embodiment, therefore, the invention provides a method of assigning a course of therapy to an individual having a condition, said method comprising classifying the individual into one of a plurality of subsets

of a condition, wherein a plurality of informative genes has been identified for at least one of said subsets; and assigning a course of therapy known or suspected to be effective for treating the subset of the condition associated with those genes. In a specific embodiment, said condition is breast cancer, said patient subset is ER+, ER/AGE high status, and said course of therapy comprises the administration of one or more compounds known or suspected to be effective at arresting the cell cycle. In a more specific embodiment, said one or more compounds comprises taxol or a vinca alkaloid.

[0128] Of course, any course of therapy selected or assigned on the basis of the above phenotypes and gene expression may be supplemented by other treatments or courses of therapy relevant to or known or suspected to be effective in the treatment of the condition. For example, the treatment of breast cancer may additionally comprise surgery, either tissue-preserving or radical, radiation treatment, chemotherapy other than that suggested by gene expression analysis, or any other therapy or treatment known or suspected to be effective.

### 5.7 CLINICAL TRIALS

The method of the present invention may also be used to assign individuals to categories within a clinical trial. For example, individuals may be distinguished according to a characteristic of a condition, such as prognosis, and results of the trial correlated with prognosis. In a specific example, the condition is breast cancer, and the characteristic is prognosis, *i.e.*, expected reoccurrence or non-reoccurrence of metastases within a given period, for example, five years, after initial diagnosis. Thus, the invention provides a method for assigning an individual to one of a plurality of categories in a clinical trial, comprising classifying the individual into one of a plurality of condition categories differentiated by at least one genotypic or phenotypic characteristic of the condition; determining the level of expression, in a sample derived from said individual, of a plurality of genes informative for said condition category; determining whether said level of expression of said plurality of genes indicates that the individual has a good prognosis or a poor prognosis; and assigning the individual to a category in a clinical trial on the basis of prognosis. In a specific embodiment, the invention provides a method of assigning an individual to a category in a breast cancer clinical trial, said method comprising: (a) classifying said individual as $ER^-$, *BRCA1*, $ER^-$, sporadic; ER+, ER/AGE high; ER+, ER/AGE low, LN+; or ER+, ER/AGE low, $LN^-$; (b) determining for said individual the level of expression of at least two genes for which markers are listed in Table 1 if said individual is classified as $ER^-$, *BRCA1*; Table 2 if said individual is classified as $ER^-$, sporadic; Table 3 if said individual is classified as ER+,

ER/AGE high; Table 4 if said individual is classified as ER+, ER/AGE low, LN+; or Table 5 if said individual is classified as ER+, ER/AGE low, LN⁻; (c) determining whether said individual has a pattern of expression of said at least two genes that correlates with a good prognosis or a poor prognosis; and (d) assigning said individual to at least one category in a clinical trial if said individual has a good prognosis, and assigning said individual to a second category in said clinical trial if said individual has a poor prognosis. In a more specific embodiment, said individual is additionally assigned to a category in said clinical trial on the basis of the classification of said individual as determined in step (a). In another more specific embodiment, said individual is additionally assigned to a category in said clinical trial on the basis of any other clinical, phenotypic or genotypic characteristic of breast cancer. In another more specific embodiment, the method additionally comprises determining in said cell sample the level of expression, relative to a control, of a second plurality of genes for which markers are not found in Tables 1-5, wherein said second plurality of genes is informative for prognosis of breast cancer, and determining from the expression of said second plurality of genes, in addition to said first plurality of genes, whether said individual has a good prognosis or a poor prognosis.

## 5.8 KITS

[0129] The present invention further provides for kits comprising the marker sets above. In a preferred embodiment, the kit contains a microarray ready for hybridization to target polynucleotide molecules. In specific embodiments, the kit may contain any of the microarrays described in detail in Section 5.5.2. The kit may additionally comprise software for the data analyses described above, as described in detail in Section 5.9.

## 5.9 COMPUTER-FACILITATED ANALYSIS

[0130] The analytic methods described in the previous sections can be implemented by use of the following computer systems and according to the following programs and methods. A computer system comprises internal components linked to external components. The internal components of a typical computer system include a processor element interconnected with a main memory. For example, the computer system can be based on an Intel 8086-, 80386-, 80486-, Pentium™, or Pentium™-based processor with preferably 32 MB or more of main memory. The computer system may also be a Macintosh or a Macintosh-based system, but may also be a minicomputer or mainframe.

[0131] The external components preferably include mass storage. This mass storage can be one or more hard disks (which are typically packaged together with the processor and

memory). Such hard disks are preferably of 1 GB or greater storage capacity. Other external components include a user interface device, which can be a monitor, together with an inputting device, which can be a "mouse", or other graphic input devices, and/or a keyboard. A printing device can also be attached to the computer.

[0132] Typically, a computer system is also linked to network link, which can be part of an Ethernet link to other local computer systems, remote computer systems, or wide area communication networks, such as the Internet. This network link allows the computer system to share data and processing tasks with other computer systems.

[0133] Loaded into memory during operation of this system are several software components, which are both standard in the art and special to the instant invention. These software components collectively cause the computer system to function according to the methods of this invention. These software components are typically stored on the mass storage device. A software component comprises the operating system, which is responsible for managing computer system and its network interconnections. This operating system can be, for example, of the Microsoft Windows® family, such as Windows 3.1, Windows 95, Windows 98, Windows 2000, or Windows NT, or may be of the Macintosh OS family, or may be UNIX, a UNIX derivative such as LINUX, or an operating system specific to a minicomputer or mainframe. The software component represents common languages and functions conveniently present on this system to assist programs implementing the methods specific to this invention. Many high or low level computer languages can be used to program the analytic methods of this invention. Instructions can be interpreted during run-time or compiled. Preferred languages include C/C++, FORTRAN and JAVA. Most preferably, the methods of this invention are programmed in mathematical software packages that allow symbolic entry of equations and high-level specification of processing, including some or all of the algorithms to be used, thereby freeing a user of the need to procedurally program individual equations or algorithms. Such packages include Mathlab from Mathworks (Natick, MA), Mathematica® from Wolfram Research (Champaign, IL), or S-Plus® from Math Soft (Cambridge, MA). Specifically, the software component includes the analytic methods of the invention as programmed in a procedural language or symbolic package.

[0134] The software to be included with the kit comprises the data analysis methods of the invention as disclosed herein. In particular, the software may include mathematical routines for marker discovery, including the calculation of similarity values between clinical categories (*e.g.*, prognosis) and marker expression. The software may also include

mathematical routines for calculating the similarity between sample marker expression and control marker expression, using array-generated fluorescence data, to determine the clinical classification of a sample.

[0135] Additionally, the software may also include mathematical routines for determining the prognostic outcome, and recommended therapeutic regimen, for a particular breast cancer patient. Such software would include instructions for the computer system's processor to receive data structures that include the level of expression of five or more of the marker genes listed in any of Tables 1-5 in a breast cancer tumor sample obtained from the breast cancer patient; the mean level of expression of the same genes in a control or template; and the breast cancer patient's clinical information, including age, lymph node status and ER status. The software may additionally include mathematical routines for transforming the hybridization data and for calculating the similarity between the expression levels for the marker genes in the patient's breast cancer tumor sample and a control or template. In a specific embodiment, the software includes mathematical routines for calculating a similarity metric, such as a coefficient of correlation, representing the similarity between the expression levels for the marker genes in the patient's breast cancer tumor sample and the control or template, and expressing the similarity as that similarity metric.

[0136] The software preferably would include decisional routines that integrate the patient's clinical and marker gene expression data, and recommend a course of therapy. In one embodiment, for example, the software causes the processor unit to receive expression data for prognosis-related genes in the patient's tumor sample, calculate a metric of similarity of these expression values to the values for the same genes in a template or control, compare this similarity metric to a pre-selected similarity metric threshold or thresholds that differentiate prognostic groups, assign the patient to the prognostic group, and, on the basis of the prognostic group, assign a recommended therapeutic regimen. In a specific example, the software additionally causes the processor unit to receive data structures comprising clinical information about the breast cancer patient. In a more specific example, such clinical information includes the patient's age, estrogen receptor status, and lymph node status.

[0137] The software preferably causes the processor unit to receive data structures comprising relevant phenotypic and/or genotypic characteristics of the particular condition of interest, and/or of an individual having that condition, and classifies the individual into a condition subset according to those characteristics. The software then causes the processor to receive values for subset-specific markers, to calculate a metric of similarity of the values associated with those markers (*e.g.*, level, abundance, activity, *etc.*) from the individual to a

control, compare this similarity metric to a pre-selected similarity metric threshold or thresholds that differentiate prognostic groups, assign the patient to a prognostic group, and, on the basis of the prognostic group, assign a recommended therapeutic regimen. In the specific example of breast cancer and a breast cancer patient, the software, in one embodiment, causes the processor unit to receive data structures comprising the patient's age, estrogen receptor status, and lymph node status, and on the basis of this data, to classify the patient into one of the following patient subsets: $ER^-$, sporadic; $ER^-$, *BRCA1*; ER+, AR/AGE high; ER+, ER/AGE low, LN+; or ER+, ER/AGE low, $LN^-$. The software then causes the processor to receive expression values for subset-specific prognosis-informative gene expression in the patient's tumor sample, calculate a metric of similarity of these expression values to the values for the same genes in a patient subset-specific template or control, compare this similarity metric to a pre-selected similarity metric threshold or thresholds that differentiate prognostic groups, assign the patient to the prognostic group, and, on the basis of the prognostic group, assign a recommended therapeutic regimen.

[0138] Where the control is an expression template comprising expression values for marker genes within a group of patients, *e.g.*, breast cancer patients, the control can comprise either hybridization data obtained at the same time (*i.e.*, in the same hybridization experiment) as the patient's individual hybridization data, or can be a set of hybridization or marker expression values stores on a computer, or on computer-readable media. If the latter is used, new patient hybridization data for the selected marker genes, obtained from initial or follow-up tumor samples, or suspected tumor samples, can be compared to the stored values for the same genes without the need for additional control hybridizations. However, the software may additionally comprise routines for updating the control data set, *e.g.*, to add information from additional breast cancer patients or to remove existing members of the control data set, and, consequently, for recalculating the average expression level values that comprise the template. In another specific embodiment, said control comprises a set of single-channel mean hybridization intensity values for each of said at least five of said genes, stored on a computer-readable medium.

[0139] Clinical data relating to a breast cancer patient, or a patient having another type of condition, and used by the computer program products of the invention, can be contained in a database of clinical data in which information on each patient is maintained in a separate record, which record may contain any information relevant to the patient, the patient's medical history, treatment, prognosis, or participation in a clinical trial or study, including

expression profile data generated as part of an initial diagnosis or for tracking the progress of the condition, for example, breast cancer, during treatment.

[0140] Thus, one embodiment of the invention provides a computer program product for classifying a breast cancer patient according to prognosis, the computer program product for use in conjunction with a computer having a memory and a processor, the computer program product comprising a computer readable storage medium having a computer program mechanism encoded thereon, wherein said computer program product can be loaded into the one or more memory units of a computer and causes the one or more processor units of the computer to execute the steps of (a) receiving a first data structure comprising said breast cancer patient's age, ER status, LN status and tumor type; (b) classifying said patient as $ER^-$, sporadic; $ER^-$, *BRCA1*; ER+, ER/AGE high; ER+, ER/AGE low, LN+; or ER+, ER/AGE low, $LN^-$; (c) receiving a first data structure comprising the level of expression of at least two genes in a cell sample taken from said breast cancer patient wherein markers for said at least two genes are listed in Table 1 if said patient is classified as $ER^-$, sporadic; Table 2 if said patient is classified as $ER^-$, sporadic; Table 3 if said patient is classified as ER+, ER/AGE high; Table 4 if said patient is classified as ER+, ER/AGE low, LN+; or Table 5 if said patient is classified as ER+, ER/AGE high, $LN^-$; (d) determining the similarity of the level of expression of said at least two genes to control levels of expression of said at least two genes to obtain a patient similarity value; (e) comparing said patient similarity value to selected first and second threshold values of similarity of said level of expression of said genes to said control levels of expression to obtain first and second similarity threshold values, respectively, wherein said second similarity threshold indicates greater similarity to said control levels of expression than does said first similarity threshold; and (f) classifying said breast cancer patient as having a first prognosis if said patient similarity value exceeds said first and said second threshold similarity values, a second prognosis if said patient similarity value exceeds said first threshold similarity value but does not exceed said second threshold similarity value, and a third prognosis if said patient similarity value does not exceed said first threshold similarity value or said second threshold similarity value. In a specific embodiment of said computer program product, said first threshold value of similarity and said second threshold value of similarity are values stored in said computer. In another more specific embodiment, said first prognosis is a "very good prognosis," said second prognosis is an "intermediate prognosis," and said third prognosis is a "poor prognosis," and wherein said computer program mechanism may be loaded into the memory and further cause said one or more processor units of said computer to execute the step of assigning said breast cancer

patient a therapeutic regimen comprising no adjuvant chemotherapy if the patient is lymph node negative and is classified as having a good prognosis or an intermediate prognosis, or comprising chemotherapy if said patient has any other combination of lymph node status and expression profile. In another specific embodiment, said computer program mechanism may be loaded into the memory and further cause said one or more processor units of the computer to execute the steps of receiving a data structure comprising clinical data specific to said breast cancer patient. In a more specific embodiment, said single-channel hybridization intensity values are log transformed. The computer implementation of the method, however, may use any desired transformation method. In another specific embodiment, the computer program product causes said processing unit to perform said comparing step (e) by calculating the difference between the level of expression of each of said genes in said cell sample taken from said breast cancer patient and the level of expression of the same genes in said control. In another specific embodiment, the computer program product causes said processing unit to perform said comparing step (e) by calculating the mean log level of expression of each of said genes in said control to obtain a control mean log expression level for each gene, calculating the log expression level for each of said genes in a breast cancer sample from said breast cancer patient to obtain a patient log expression level, and calculating the difference between the patient log expression level and the control mean log expression for each of said genes. In another specific embodiment, the computer program product causes said processing unit to perform said comparing step (e) by calculating similarity between the level of expression of each of said genes in said cell sample taken from said breast cancer patient and the level of expression of the same genes in said control, wherein said similarity is expressed as a similarity value. In more specific embodiment, said similarity value is a correlation coefficient. The similarity value may, however, be expressed as any art-known similarity metric.

[0141] In an exemplary implementation, to practice the methods of the present invention, a user first loads experimental data into the computer system. These data can be directly entered by the user from a monitor, keyboard, or from other computer systems linked by a network connection, or on removable storage media such as a CD-ROM, floppy disk (not illustrated), tape drive (not illustrated), ZIP® drive (not illustrated) or through the network. Next the user causes execution of expression profile analysis software which performs the methods of the present invention.

[0142] In another exemplary implementation, a user first loads experimental data and/or databases into the computer system. This data is loaded into the memory from the storage

media or from a remote computer, preferably from a dynamic geneset database system, through the network. Next the user causes execution of software that performs the steps of the present invention.

[0143] Additionally, because the data obtained and analyzed in the software and computer system products of the invention are confidential, the software and/or computer system comprises access controls or access control routines, such as

[0144] Alternative computer systems and software for implementing the analytic methods of this invention will be apparent to one of skill in the art and are intended to be comprehended within the accompanying claims. In particular, the accompanying claims are intended to include the alternative program structures for implementing the methods of this invention that will be readily apparent to one of skill in the art.

## 6. EXAMPLE: IDENTIFICATION OF PHENOTYPIC SUBSETS AND INFORMATIVE GENESETS FOR EACH

*Materials and Methods*

Tumor Samples:

[0145] 311 cohort samples were collected from breast cancer patients. Selection criteria for sporadic patients (*i.e.*, those not identified as having a *BRCA1*-type tumor; $n = 291$) included: primary invasive breast carcinoma less than 5 cm (T1 or T2); no axillary metastases (N0); age at diagnosis of less than 55 years; calendar year of diagnosis 1983-1996; and no previous malignancies. All patients were treated by modified radical mastectomy or breast-conserving treatment. *See* van't Veer *et al.*, *Nature* 415:530 (2002). Selection criteria for hereditary (*i.e.*, *BRCA1*-type; $n = 20$) tumors included: carriers of germline mutation in *BRCA1* or *BRCA2*, and primary invasive breast carcinoma. van't Veer, *supra*. Additionally, for development of a classifier for the BRCA1 group, 14 BRCA1 samples previously identified (*see* van't Veer, *supra*) were added to the 20 BRCA1 type samples to increase sample size. Those 14 samples also satisfy the conditions that they are ER negative and age less than 55 years old.

[0146] Data analysis:

[0147] Sample sub-grouping: As shown in FIG. 1, tumor samples were first divided into ER+ and $ER^-$ branches since this is the dominant gene expression pattern. In the $ER^-$ branch, the samples were further divided into "BRCA1 mutation like" and "Sporadic like" categories using the expression templates and 100 genes previously identified as optimal for determining *BRCA1* status. *See* van't Veer *et al.*, *Nature* 415:530 (2002). In the ER+

category, samples were divided by ER vs. age distribution (see below) into two groups, "ER/AGE low" and "ER/AGE high." Within the "ER/AGE low" group, samples were further divided according to the lymph node status into two sub-groups: lymph node negative (0 lymph nodes; LN–) and positive (> 0 lymph nodes; LN+) group.

[0148] The result of these divisions was five distinctive sub-groups: "$ER^-$, sporadic" ($n$ = 52), "$ER^-$, BRCA1" ($n$ = 34), "ER+, ER/AGE high" ($n$ = 83), "ER+, ER/AGE low, $LN^-$" ($n$ = 81), and "ER+, ER/AGE low, LN+" ($n$ = 75). A few samples with a specific ER vs. age distribution in "ER+, ER/AGE low, LN+" group were further excluded to develop a classifier, see below for details.

[0149] Estrogen receptor level: Estrogen receptor gene expression level was measured by a 60mer oligo-nucleotide on a microarray. Since every individual sample was compared to a pool of all samples, the ratio to pool was used to measure the relative level. A threshold of –0.65 on $\log_{10}$(ratio) was used to separate the ER+ group from $ER^-$ group. *See* van't Veer *et al., Nature* 415:530 (2002).

[0150] Grouping by ER vs. age distribution: Samples were not uniformly distributed in ER vs. age space among the ER+ samples (FIG. 2). First, it appeared that the ER level increases with age, as there were few samples from young individuals having a high ER expression level. For example, in the 35 to 40 years age group, samples having a log(ratio) of ER > 0.2 are relatively few as compared to the 40 to 45 age group. In the set of samples used, the 40 < age ≤ 45 group contains 30 samples having log(ratio) ER values between –0.2 to 0.2, and 28 samples having values greater than 0.2, whereas the 35 < age ≤ 40 group includes 24 samples with values between –0.2 to 0.2, but only 6 samples with values of greater than 0.2 (Fisher's exact test P-value: 1%). The increasing ER level with age may simply due to the fact that estrogen levels decrease with age, and the estrogen receptor level rises in compensation.

[0151] There also appear to be at least two groups of patients, as indicated by the solid line separating the two in FIG. 2A. A bimodality test of the separation indicated by the solid line yielded P-value < $10^{-4}$. Each of these two groups has its own trend between the ER level and age. The solid line can be approximated by ER = 0.1(age – 42.5). Patients having values above the solid line are referred to as the "ER/AGE high" group, and the patients below the line as the "ER/AGE low" group.

*Prognosis in each group*:

[0152] Feature selection and performance evaluation: For the prognosis in each group, non-informative genes were filtered in each group of patients. Specifically, only genes with $|\log_{10}(\text{ratio})| > \log_{10}(2)$ and P-value (for log(ratio) ≠ 0) < 0.01 in more than 3 experiments

were kept. This step removed all genes that never had any significant change across all samples. The second step used a leave-one-out cross validation (LOOCV) procedure to optimize the number of reporter genes (features) in the classifier and to estimate the performance of the classifier in each group. The feature selection was included inside the loop of each LOOCV process. The final "optimal" reporter genes were selected using all of the "training samples" as the result of "re-substitution" because one classifier was needed for each group.

[0153] Selection of training samples: Only the samples from patients who had metastases within 5 years of initial diagnosis (3 years for "$ER^-$, sporadic" samples; *i.e.*, the "poor outcome" group), or who were metastases-free with more than 5 years of follow-up time (*i.e.*, the "good outcome" group, were used as the training set. Because the average expression levels for informative genes among patients who were metastasis-free, or who had early metastases, were used as expression templates for prediction, the training samples for the ER+ samples were further limited to those samples that could also be correctly classified by the first round of LOOCV process. For the "$ER^-$, sporadic" samples, no such iteration was done because no improvement was observed. For the "$ER^-$, BRCA1" samples, an iteration was done, but the training samples in the second iteration were limited to the correctly predicted good outcome samples from the first round of LOOCV, and all the poor outcome samples with metastases time less than 5 years. Further limitation of the poor outcome samples was not performed because of the small number of poor samples and the absence of improvement by such limitation. In the first round of LOOCV, except for the "$ER^-$, sporadic" group, the number of features was fixed at 50 genes. A patient was predicted to have a favorable outcome, that is, no metastases within five years of initial diagnosis, if the expression of the reporter genes in a sample from the individual was more similar to the "average good profile" than the "average poor profile", and a poor outcome, that is, a metastasis within five years, if the expression of the reporter genes in the sample was more similar to the "average poor profile" than the "average good profile".

[0154] The justification for such an iteration operation is threefold. First, biologically, there are always a few individuals with specific reasons (different from the vast majority) to stay metastases free or to develop metastases. Second, statistically, most groups of patients include outliers that don't follow the distribution of the majority of samples. Third, methodologically, the iteration operation is very similar to the idea of "boosting", but instead of increasing the weights of the samples predicted wrong, emphasis is placed on the well behaved samples for selecting features and training the classifier. Since this process was

used to select "training samples", and the performance was evaluated using the LOOCV (including the feature selection) after the training sample being fixed, there is no issue of over-fitting involved in our procedures. This method of iteration is thus more likely to reveal the dominant mode to metastases within each group.

[0155] Error rate and odds ratio, threshold in the final LOOCV: Unless otherwise stated, the error rate is the average error rate from two populations: (1) the number of poor outcome samples misclassified as good outcome samples, divided by the total number of poor outcome samples; and (2) the total number of good outcome samples misclassified as poor outcome samples, divided by the total number of good samples. Two odds ratios are reported for a given threshold: (1) the overall odds ratio and (2) the 5 year odds ratio. The 5 year odds ratio was calculated from samples from individuals that were metastases free for more than five years, and who experienced metastasis within 5 years. The threshold was applied to **cor1 – cor2**, where "cor1" stands for the correlation to the "average good profile" in the training set, and "cor2" stands for the correlation to the "average poor profile" in the training set.

[0156] The threshold in the final round of LOOCV was defined using the following steps: (1) For each of the N sample *i* left out for training, features based on the training set were selected. (2) Given a feature set, an incomplete LOOCV with N-1 samples was performed (only the "average poor profile" and "average good profile" is varied depending on whether the left out sample is in the training set or not). (3) The threshold based on the minimum error rate from N-1 samples was determined, and that threshold was assigned to sample *i* in step (1). (4) The median threshold from all N samples was taken, and designated the final threshold. FIGS. 3-7 present detailed information about classifiers for the 5 groups: "ER$^-$, sporadic", "ER$^-$, BRCA1", "ER+, ER/age high", "ER+, ER/age low, LN$^-$", "ER+, ER/age low, LN+". Tables 1-5 (*see* Section 5.3) list the final optimal reporter genes for each of the 5 classifiers for each of the five patient subsets. Table 6, below, summarizes the performance of each of the five classifiers together with thresholds used in each classifier.

Table 6. Performance of classifiers for each patient subset.

| Classifier | Optimal # of Genes | (C1-C2) Threshold | Metastasis Free | # of Samples | TP | FP | FN | TN | Odds Ratio | 95% C.I. |
|---|---|---|---|---|---|---|---|---|---|---|
| ER+, ER/AGE high | 50 | 1.22 | Overall | 83 | 31 | 14 | 5 | 33 | 14.61 | 4.71-45.36 |
| | | | 5 year | 71 | 24 | 11 | 3 | 33 | 24.00 | 6.03-95.46 |
| ER+, ER/AGE low, LN- | 65 | 0.38 | Overall | 81 | 14 | 6 | 6 | 55 | 21.39 | 5.98-76.52 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 5 year | 73 | 11 | 4 | 5 | 53 | 29.15 | 6.73-126.33 |
| **ER+, ER/AGE low, LN+** | 50 | -0.12 | Overall | 56 | 7 | 4 | 6 | 39 | 11.38 | 2.54-50.94 |
| | | | 5 year | 48 | 5 | 4 | 3 | 36 | 15.00 | 2.57-87.64 |
| **ER-, sporadic** | 20 | -0.01 | Overall | 52 | 18 | 7 | 7 | 29 | 7.35 | 2.16-25.04 |
| | | | 5 year | 45 | 16 | 5 | 6 | 18 | 9.60 | 2.45-37.58 |
| **ER-, BRCA1** | 10 | -0.37 | Overall | 34 | 6 | 3 | 3 | 22 | 14.67 | 2.34-92.11 |
| | | | 5 year | 22 | 6 | 1 | 3 | 12 | 24.00 | 2.04-282.68 |

TP: True positive
FP: False positive
FN: False negative
TN: True negative

[0157] Classification method: All classifiers described herein, feature selection and optimization were included inside the LOOCV loop. Classifier performance was based on the LOOCV results. The profile based on the selected features from each patient was compared to the "average good profile" and "average poor profile" (by correlation) to determine its predicted outcome.

[0158] Correlation calculation: The correlation between each gene's expression log(ratio) and the endpoint data (final outcome) was calculated using the Pearson's correlation coefficient. The correlation between each patient's profile and the "average good profile" and "average poor profile" is the cosine product (no mean subtraction).

*Results:*

[0159] We employed the comprehensive prognosis strategy on microarray expression profiles of 311 patients diagnosed before age 55 that were all part of previous studies establishing and validating a 70-gene prognosis profile. *See* van 't Veer *et al.*, *Nature* 415:530 (2002); van de Vijver *et al.*, *N. Engl. J. Med.* 347:1999 (2002). In addition, 14 known *BRCA1* samples from the *Nature* study were included in defining the prognosis classifier for the *BRCA1* group. The overview of the stratifications is shown in FIG. 1. In each of the patient subsets, prognosis classifiers were developed and performance was evaluated by leave-one-out cross-validation. The biological make up of each of the classifiers was also examined.

[0160] During the process to decide whether a particular clinical parameter should be used for the next stratification, our objectives were twofold: (1) identification of homogeneous prognosis patterns; and/or (2) improved prognosis in the subsets. There is a subtle balance

between these two objectives because smaller groups will likely lead to uniform patterns within the group but have increasingly limited predictive power. With the exception of the *BRCA1* subset, each group in our stratification contained 50 or more samples.

[0161] The first layer of stratification was based on the estrogen receptor level. We and others previously observed that estrogen receptor expression has a dominant effect on overall gene expression in breast cancer as seen in hierarchical clustering. van 't Veer *et al., Nature* 415:530 (2002); Perou *et al., Nature* 406:747 (2000); Gruvberger *et al., Cancer Res.* 61:5979 (2001). In our previous analysis up to 2500 genes are significantly correlated with ER expression levels in tumor. van 't Veer *et al., Nature* 415:530 (2002). According to the threshold defined previously (van de Vijver *et al., N. Engl. J. Med.* 347:1999 (2002)), samples were first divided into two groups according to the estrogen receptor level as measured by the oligo probe (accession number: NM_000125) on the array; samples with log(ratio) > –0.65 belong to the ER+ group, and the rest belong to $ER^-$ group). This resulted in 239 samples in the ER+ group and 72 samples in the $ER^-$ group.

[0162] In the ER+ branch we observed that when displaying ER expression level as a function of age, at least two subgroups appear to exist. (In general, any bimodality in the clinical data is useful.) We therefore decided to stratify the tumors according this bimodality (*see* FIG. 2). The group of ER+ patients having a high ER/AGE ratio was designated the "ER/AGE high" group (83 samples), and the remaining group of patients was designated "ER/AGE low" group (156 samples).

[0163] Within the "ER/age high" group, we identified a group of prognosis reporter genes that highly correlated with the outcome (*see* Table 3). Moreover, the expression of these genes appeared to be very homogeneous, as indicated by high similarity in expression among those genes. *See* FIG 2A. Leave-one-out cross validation including reporter selection yielded an odds ratio of 14.6 (95%CI: 4.7-45.4) and 5 year odds ratio of 24.0 (95%CI: 6.0-95.5). Examination of those reporter genes reveals they are mostly the cell cycle genes which are highly expressed in the poor outcome tumors. It is worth noting that even though this group includes LN+ and LN– individuals, and mixed treatment, the incidence of distant metastases is predicted by a biologically uniform set of genes, possibly indicating that proliferation is the prime driving force for disease progression. Also even though variation in these genes is observed in other tumor subgroups this is generally not correlated with outcome in those settings (see below).

[0164] In the "ER/age low" group, no predictive pattern was found in the whole group; thus, the samples were further stratified into LN– (81 samples, referred to as "ER/age low $LN^-$") and LN+ (75 samples, referred to as "ER/age low LN+") group.

[0165] Within the "ER/age low $LN^-$" group, a group of genes was identified that was uniformly co-regulated, and which correlated with the outcome. Leave-one-out cross-validation (including feature selection) yielded an odds ratio of 21.4 (95% CI: 6.0-76.5) and 5 year odds ratio of 29.2 (95% CI: 6.7-126.3). This group of genes is also enriched for individual biological functions (see below).

[0166] For the "ER/age low LN+" subset, an informative set of genes (*see* Table 4) was obtained after exclusion of several samples from older individuals having low ER levels. These samples are indicated in FIG. 2A as those lying below the dashed line (approximated as ER < 0.1*(age-50). 56 samples remained after the exclusion. This sample set allowed the identification of a group of genes with a highly homogeneous pattern that is useful for prognosis (overall odds ratio: 11.4 (2.5-50.9), 5 year odds ratio: 15.0 (2.6-87.6)). This suggests again that ER vs. age is an important combination for stratifying breast cancer patients. The reporter genes involved in this classifier also correlated with the clinical measure of the degree of lymphocytic infiltration (data not shown). The prediction in this group is not as strong as other positive groups, which may indicate the primary tumor carries weaker information about the metastases for this group of patients, and the metastases may be started from or influenced by tumors already in lymph nodes.

[0167] In the $ER^-$ branch, because a portion of the samples are "*BRCA1*-like," it is natural to divide the samples into "*BRCA1*-like" and "sporadic like". To perform the classification, the *BRCA1*/sporadic tumor type classifier described in Roberts *et al.*, "Diagnosis and Prognosis of Breast Cancer Patients," International Publication No. WO 02/103320, which is hereby incorporated by reference in its entirety, to segregate the $ER^-$ cohort samples. 52 out of the 72 $ER^-$ samples were found to be "sporadic like" and 20 were found to be "*BRCA1*-like". Interestingly, the "sporadic like" group is enriched for erbb2 mutations (data not shown).

[0168] Within the "$ER^-$, sporadic" group, no homogeneous prognosis pattern was identified; however, 20 genes were identified that are highly predictive of the tumor outcome (*see* Table 2). Leave-one-out cross-validation including feature selection yielded an odds ratio of 7.4 (95% CI 2.2-25.0) and 5 year odds ratio 9.6 (2.5 – 37.6). This result represents a significant improvement in prognosis compared to the previously-identified 70 gene prognosis classifier (*see* Roberts *et al.*, International Publication No. WO 02/103320; van 't Veer *et al.*, *Nature* 415:530 (2002)) which has no within-group prognostic power for the $ER^-$ patient subset.

The fact that 20 genes predict outcome and that there is no homogeneous (and apparent biological) pattern in this group probably indicates multiple mechanisms of metastasis in this group. Gene annotation indicates that genes included may be involved in invasion, energy metabolism and other functions.

[0169] For the "ER$^-$, *BRCA1*-like" group, we added 14 BRCA1 mutation carrier samples from our previous study to increase the number of samples. Those 14 extra samples also satisfy our selection criteria: ER negative and age less than 55 years. The leave-one-out cross validation process identified 10 genes that are predictive of final outcomes. The overall odds ratio is 14.7 (95% CI: 2.3-92.1) and the 5 year odds ratio is 24.0 (95% CI: 2.0-282.7).

[0170] Because no homogeneous gene expression patterns were found in ER$^-$ branch, the predictive power of those genes was further validated. One means of further validation was to review the different classifier gene sets for biological interpretations and to identify genes within each classifier that gave indications as to the origins of the tumors.

[0171] The "ER+, ER/AGE high" group yielded a classifier highly enriched for cell cycle genes with both G1/S and G2/M phases represented. In this group, over-expression of 46 of the 50 genes is associated with disease progression including all the known cell cycle genes. This is consistent with rapid growth being the determinant of metastatic potential. Four genes in this classifier are anti-correlated with outcome and cell cycle. One of these genes encodes follistatin, which binds to and inhibits activin and other members of the TGFβ family (Lin *et al.*, *Reproduction* 126:133 (2003)), the members of which have many functions, including growth stimulation. Tumor grade also accurately predicts metastatic potential in this group (overall odds ratio: 5.9, 95% CI: 2.0-18.0, 5 year odds ratio: 12.5, 95% CI: 2.6-59.3) and is also correlated with the expression level of these genes, which is consistent with rate of growth being the primary determinant of disease progression. This set of genes has a significantly lower correlation with outcome in the other patient subsets, even though coordinate and similarly variable expression is seen. For example, many tumors in the "ER$^-$, sporadic" group have high cell cycle and low FST expression, but the expression of these genes in these groups is minimally correlated with outcome, indicating that growth is not the primary determinant of outcome here (*see* FIGS. 8A and 8B).

[0172] The ER+, ER/AGE low, LN$^-$ group yielded a classifier rich in both genes for glycolytic enzymes (12 of 56) and genes induced by hypoxia and/or angiogenesis (14 of 56) with 5 genes falling into both categories. These genes are positively correlated with poor outcome, implying that energy metabolism (glycolysis), angiogenesis and adaptation to hypoxia are critical pathways in this subgroup of tumors. None of these genes appear in the

classifiers for the other patient subsets, and there is a much reduced predictive value of these genes in the other tumors, even though coordinate and similarly variable expression is seen (*see* FIG. 8C and 8D).

[0173] The implication of the above analyses is that certain well known functions (growth, angiogenesis, energy metabolism) are important in certain tumor types and not in others, and therefore therapies that target these functions will be likely be similarly effective in some tumor subgroups and not in others. For example therapies that target cell cycle progression, such as taxol or the vinca alkaloids, may be optimally effective in the ER+, ER/AGE high group, where overexpression of cell cycle genes predominates in the classifier. In contrast, tumor subgroups in which variation in cell cycle expression is not correlated with outcome may be less sensitive to taxol or the vinca alkaloids.

[0174] The "comprehensive prognosis" approach significantly improves the prediction error rate when compared with 70 gene classifier (Table 7). To make the comparison fair, we listed two sets of results from the 70 gene classifier. The first results from the use of the same threshold applied to all the patient subsets (threshold previously optimized for false negative rate); the second one results from the use of a threshold optimized for each patient subset (optimized for average error rate). The comprehensive approach lowered the error rate by at least 6%.

Table 7. Average error rate for the patient subset approach compared with the previously-described 70 gene classifier.

| Prognosis method | over all error rate | 5 year error rate |
|---|---|---|
| 70 gene, fix thresh | 30.90% | 25.70% |
| 70 gene, opt thresh | 28.60% | 27.60% |
| Comprehensive | 21.50% | 19.30% |

Fix thresh: use of a fixed threshold in the classifier as previously determined.
Opt threshold: use of a threshold optimized for each sub-group. For the "ER/Age low, LN+" subgroup, 56 samples used for developing the classifier were included here, resulted in 306 samples in total.

## 7. REFERENCES CITED

[0175] All references cited herein are incorporated herein by reference in their entirety and for all purposes to the same extent as if each individual publication or patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety for all purposes.

[0176] Many modifications and variations of the present invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited only by the terms of the appended claims along with the full scope of equivalents to which such claims are entitled.

What is claimed is:

1. A method of identifying a set of informative genes or markers for a condition comprising a plurality of phenotypic or genotypic characteristics, comprising:

(a) classifying each of a plurality of samples or individuals on the basis of one or more phenotypic or genotypic characteristics of said condition into a plurality of first classes; and

(b) identifying within each of said first classes a first set of genes or markers informative for said condition

wherein said first set of genes or markers within each of said first classes is unique to said class relative to other first classes.

2. The method of claim 1, which further comprises additionally classifying into a plurality of second classes said samples or individuals in at least one of said first classes on the basis of a phenotypic or genotypic characteristic different that that used in said classifying step (a); and identifying within at least one of said second classes a second set of informative genes or markers, wherein said second set of informative genes or markers within each of said second classes is unique to said second class relative to other first and second classes.

3. A method of identifying a set of informative genes or markers for a condition comprising a plurality of phenotypic or genotypic characteristics, comprising:

(a) classifying each of a plurality of samples or individuals on the basis of one or more phenotypic or genotypic characteristics into a plurality of first classes;

(b) classifying at least one of said first classes into a plurality of second classes on the basis of phenotypic or genotypic characteristic different than that used in said classifying step (a); and

(c) identifying within at least one of said first classes or said second classes a set of genes or markers informative for said condition,

wherein said second set of genes or markers is unique to said class relative to other first and second classes.

4. A method of identifying a set of informative genes or markers for a condition comprising a plurality of phenotypic or genotypic characteristics, comprising:

(a) selecting a first characteristic from said plurality of phenotypic or genotypic characteristics;

(b) identifying at least two first condition classes differentiable by said first characteristic;

(c) selecting a plurality of individuals classifiable into at least one of said first condition classes; and

(d) identifying in samples derived from each of said plurality of individuals a set of genes or markers informative for said condition within said at least one of said first condition classes.

5. A method of classifying an individual with a condition as having a good prognosis or a poor prognosis, comprising:

(a) classifying said individual into one of a plurality of patient classes, said patient classes being differentiated by one or more phenotypic, genotypic or clinical characteristics of said condition;

(b) determining the level of expression of a plurality of genes or their encoded proteins in a cell sample taken from the individual relative to a control, said plurality of genes or their encoded proteins comprising genes or their encoded proteins informative for prognosis of the patient class into which said individual is classified; and

(c) classifying said individual as having a good prognosis or a poor prognosis on the basis of said level of expression.

6. The method of claim 5, wherein said condition is cancer, said good prognosis is the non-occurrence of metastases within five years of initial diagnosis, and said poor prognosis is the occurrence of metastases within five years of initial diagnosis.

7. The method of claim 5, wherein said control is the average level of expression of each of said plurality of genes or their encoded proteins across a plurality of samples derived from individuals identified as having a poor prognosis.

8. The method of claim 7, in which said classifying step (c) is carried out by a method comprising comparing the level of expression of each of said plurality of genes or their encoded proteins to said average level of expression of each corresponding gene or its encoded protein in said control, and classifying said individual as having a poor prognosis if said level of expression correlates with said average level of expression of each of said genes or their encoded proteins in said control more strongly than would be expected by chance.

9. The method of claim 5, wherein said control is the average level of expression of each of said plurality of genes or their encoded proteins across a plurality of samples derived from individuals identified as having a good prognosis.

10. The method of claim 9, in which said classifying in step (c) is carried out by a method comprising comparing the level expression of each of said plurality of genes or their

encoded proteins to said average level of expression of each corresponding gene or its encoded protein in said control, and classifying said individual as having a good prognosis if said level of expression correlates with said average level of expression of each of said genes or their encoded proteins in said control more strongly than would be expected by chance.

11. The method of claim 5, wherein said plurality of patient classes comprises $ER^-$, *BRCA1* individuals; $ER^-$, sporadic individuals; ER+, ER/AGE high individuals; ER+, ER/AGE low, LN+ individuals; and ER+, ER/AGE low, $LN^-$ individuals.

12. A method of classifying a breast cancer patient as having a good prognosis or a poor prognosis comprising:

(a) classifying said breast cancer patient as $ER^-$, *BRCA1*; $ER^-$, sporadic; ER+, ER/AGE high; ER+, ER/AGE low, LN+; or ER+, ER/AGE low, $LN^-$;

(b) determining the level of expression of a first plurality of genes in a cell sample taken from said breast cancer patient relative to a control, said first plurality of genes comprising two of the genes corresponding to the markers in Table 1 if said breast cancer patient is classified as $ER^-$, *BRCA1*; in Table 2 if said breast cancer patient is classified as $ER^-$ sporadic; in Table 3 if said breast cancer patient is classified as ER+, ER/AGE high; in Table 4 if said breast cancer patient is classified as ER+. ER/AGE low, LN+; or in Table 5 if said breast cancer patient is classified as ER+, ER/AGE low, $LN^-$; and

(c) classifying said breast cancer patient as having a good prognosis or a poor prognosis on the basis of the level of expression of said first plurality of genes,

wherein said breast cancer patient is "ER/AGE high" if the ratio of the $\log_{10}$(ratio) of ER gene expression to age exceeds a predetermined value, and "ER/AGE low" if the ratio of the $\log_{10}$(ratio) of ER gene expression to age does not exceed said predetermined value.

13. The method of claim 12, wherein said control is the average level of expression of each of said plurality of genes in a plurality of samples derived from $ER^-$, *BRCA1* individuals, if said breast cancer patient is $ER^-$, *BRCA1*; the average level of expression of each of said plurality of genes in a plurality of samples derived from $ER^-$, sporadic individuals if said breast cancer patient is $ER^-$, sporadic; the average level of expression of each of said plurality of genes in a plurality of samples derived from ER+, ER/AGE high individuals, if said breast cancer patient is ER+, ER/AGE high; the average level of expression of each of said plurality of genes in a plurality of samples derived from ER+, ER/AGE low, LN+ individuals where said breast cancer patient is ER+, ER/AGE low, LN+; or the average level of expression of each of said plurality of genes in a plurality of

samples derived from ER+, ER/AGE low, $LN^-$ individuals where said breast cancer patient is ER+, ER/AGE low, $LN^-$.

14. The method of claim 13, wherein each of said individuals has a poor prognosis.

15. The method of claim 13, wherein each of said individuals has a good prognosis.

16. The method of claim 14, wherein said classifying step (c) is carried out by a method comprising comparing the level of expression of each of said plurality of genes or their encoded proteins in a sample from said breast cancer patient to said control, and classifying said breast cancer patient as having a poor prognosis if said level of expression correlates with said average level of expression of the corresponding genes or their encoded proteins in said control more strongly than would be expected by chance.

17. The method of claim 12, wherein said predetermined value of ER is calculated as $ER = 0.1(AGE - 42.5)$, wherein AGE is the age of said individual.

18. The method of claim 12, wherein said individual is $ER^-$, *BRCA1*, and said plurality of genes comprises two of the genes for which markers are listed in Table 1.

19. The method of claim 12, wherein said individual is $ER^-$, *BRCA1*, and said plurality of genes comprises all of the genes for which markers are listed in Table 1.

20. The method of claim 12, wherein said individual is $ER^-$, sporadic, and said plurality of genes comprises two of the genes for which markers are listed in Table 2.

21. The method of claim 12, wherein said individual is $ER^-$, sporadic, and said plurality of genes comprises all of the genes for which markers are listed in Table 2.

22. The method of claim 12, wherein said individual is ER+, ER/AGE high, and said plurality of genes comprises two of the genes for which markers are listed in Table 3.

23. The method of claim 12, wherein said individual is ER+, ER/AGE high, and said plurality of genes comprises all of the genes for which markers are listed in Table 3.

24. The method of claim 12, wherein said individual is ER+, ER/AGE low, LN+, and said plurality of genes comprises two of the genes for which markers are listed in Table 4.

25. The method of claim 12, wherein said individual is ER+, ER/AGE low, LN+, and said plurality of genes comprises all of the genes for which markers are listed in Table 4.

26. The method of claim 12, wherein said individual is ER+, ER/AGE low, $LN^-$, and said plurality of genes comprises two of the genes for which markers are listed in Table 4.

27. The method of claim 12, wherein said individual is ER+, ER/AGE low, $LN^-$, and said plurality of genes comprises all of the genes for which markers are listed in Table 4.

28. The method of claim 12, further comprising determining in said cell sample the level of expression, relative to a control, of a second plurality of genes for which markers are not found in Tables 1-5, wherein said second plurality of genes is informative for prognosis.

29. A method for assigning an individual to one of a plurality of categories in a clinical trial, comprising:

(a) classifying said individual as $ER^-$, *BRCA1*, $ER^-$, sporadic; ER+, ER/AGE high; ER+, ER/AGE low, LN+; or ER+, ER/AGE low, $LN^-$;

(b) determining for said individual the level of expression of at least two genes for which markers are listed in Table 1 if said individual is classified as $ER^-$, *BRCA1*; Table 2 if said individual is classified as $ER^-$, sporadic; Table 3 if said individual is classified as ER+, ER/AGE high; Table 4 if said individual is classified as ER+, ER/AGE low, LN+; or Table 5 if said individual is classified as ER+, ER/AGE low, $LN^-$;

(c) determining whether said individual has a pattern of expression of said at least two genes that correlates with a good prognosis or a poor prognosis; and

(d) assigning said individual to one category in a clinical trial if said individual has a good prognosis, and assigning said individual to a second category in said clinical trial if said individual has a poor prognosis.

30. The method of claim 29, wherein said individual is additionally assigned to a category in said clinical trial on the basis of the classification of said individual as determined in step (a).

31. The method of claim 29, wherein said individual is additionally assigned to a category in said clinical trial on the basis of any other clinical, phenotypic or genotypic characteristic of breast cancer.

32. The method of claim 29, further comprising determining in said cell sample the level of expression, relative to a control, of a second plurality of genes for which markers are not found in Tables 1-5, wherein said second plurality of genes is informative for prognosis of breast cancer, and determining from the expression of said second plurality of genes, in addition to said first plurality of genes, whether said individual has a good prognosis or a poor prognosis.

33. A microarray comprising probes complementary and hybridizable to a plurality of the genes for which markers are listed in any of Tables 1-5.

34. A microarray comprising probes complementary and hybridizable to a plurality of the genes for which markers are listed in Table 1.

35. A microarray comprising probes complementary and hybridizable to each of the genes for which markers are listed in Table 1.

36. A microarray comprising probes complementary and hybridizable to a plurality of the genes for which markers are listed in Table 2.

37. A microarray comprising probes complementary and hybridizable to each of the genes for which markers are listed in Table 2.

38. A microarray comprising probes complementary and hybridizable to a plurality of the genes for which markers are listed in Table 3.

39. A microarray comprising probes complementary and hybridizable to each of the genes for which markers are listed in Table 3.

40. A microarray comprising probes complementary and hybridizable to a plurality of the genes for which markers are listed in Table 4.

41. A microarray comprising probes complementary and hybridizable to each of the genes for which markers are listed in Table 4.

42. A microarray comprising probes complementary and hybridizable to a plurality of the genes for which markers are listed in Table 5.

43. A microarray comprising probes complementary and hybridizable to each of the genes for which markers are listed in Table 5.

44. The microarray of any of claims 33-43, wherein said probes are at least 50% of the probes on said microarray.

45. The microarray of any of claims 33-43, wherein said probes are at least 90% of the probes on said microarray.

46. The microarray of claim 33, wherein said probes are complementary and hybridizable to at least 75% of the genes for which markers are listed in Table 1; are complementary and hybridizable to at least 75% of the genes for which markers are listed in Table 2; are complementary and hybridizable to at least 75% of the genes for which markers are listed in Table 3; are complementary and hybridizable to at least 75% of the genes for which markers are listed in Table 4; and are complementary and hybridizable to at least 75% of the genes for which markers are listed in Table 5, wherein said probes, in total, are at least 50% of the probes on said microarray.

47. A kit comprising the microarray of claim 33 in a sealed container.

48. A kit comprising the microarray of claim 40 in a sealed container.

49. A kit comprising the microarray of claim 42 in a sealed container.

50. A method of identifying a set of genes informative for a condition, said condition having a plurality of phenotypic or genotypic characteristics such that samples may be categorized by at least one of said phenotypic or genotypic characteristics into at least one characteristic class, said method comprising:

(a) selecting a plurality of samples from individuals having said condition;

(b) identifying a first set of genes informative for said characteristic class using said plurality of samples;

(c) predicting the characteristic class of each of said plurality of samples;

(d) discarding samples for which said characteristic class is incorrectly predicted;

(e) repeating steps (c) and (d) at least once; and

(f) identifying a second set of genes informative for said characteristic class using samples in said plurality of samples remaining after step (e).

51. The method of claim 6, wherein said cancer is breast cancer.

52. A method for assigning an individual to one of a plurality of categories in a clinical trial, comprising:

(a) classifying the individual into one of a plurality of condition categories differentiated by at least one genotypic or phenotypic characteristic of the condition;

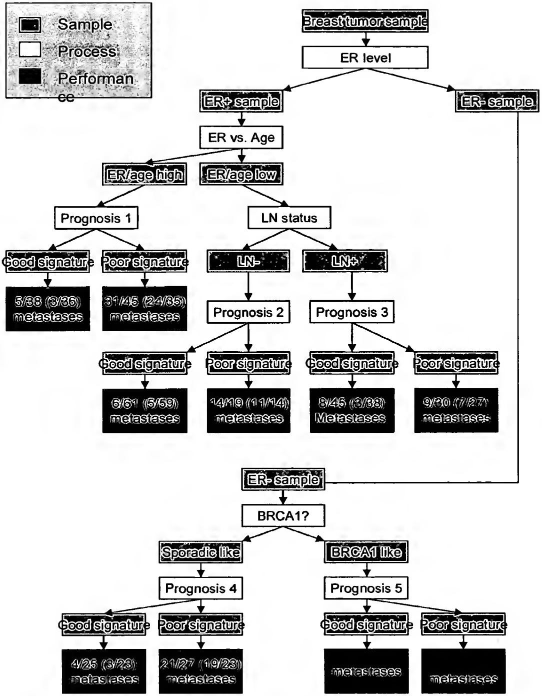
(b) determining the level of expression, in a sample derived from said individual, of a plurality of genes informative for said condition category;

(c) determining whether said level of expression of said plurality of genes indicates that the individual has a good prognosis or a poor prognosis; and

(d) assigning the individual to a category in a clinical trial on the basis of prognosis.

## ABSTRACT

[0177] The present invention provides methods for the prognosis of breast cancer, comprising classifying an individual by a plurality of phenotypic, genotypic or clinical characteristics of breast cancer into a plurality of patient subsets, and analyzing the pattern of expression of prognosis-informative genes identified for that subset in a tumor sample from the individual. The present invention also provides methods for constructing such patient subsets and of identifying prognosis-informative genesets for such subsets. The invention further provides methods of assigning a therapeutic regimen to an individual, microarrays useful for performing prognosis, kits comprising these microarrays, and computer-implementations of the methods of the invention.



Sample
Process
Performance
Breast tumor sample
ER level
ER+ sample
ER- sample
ER vs. Age
ER/age high
ER/age low
Prognosis 1
LN status
Good signature
Poor signature
LN-
LN+
5/38 (3/36) metastases
31/45 (24/35) metastases
Prognosis 2
Prognosis 3
Good signature
Poor signature
Good signature
Poor signature
6/61 (5/59) metastases
14/19 (11/14) metastases
8/45 (3/38) Metastases
9/30 (7/27) metastases
ER- sample
BRCA1?
Sporadic like
BRCA1 like
Prognosis 4
Prognosis 5
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Poor signature
Good signature
Poor signature
4/25 (3/23) metastases
21/27 (19/23) metastases
metastases
metastases


FIG. 1

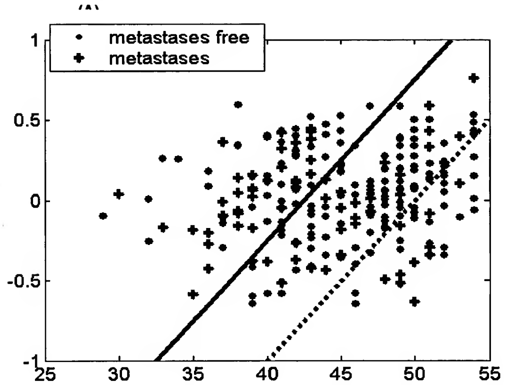
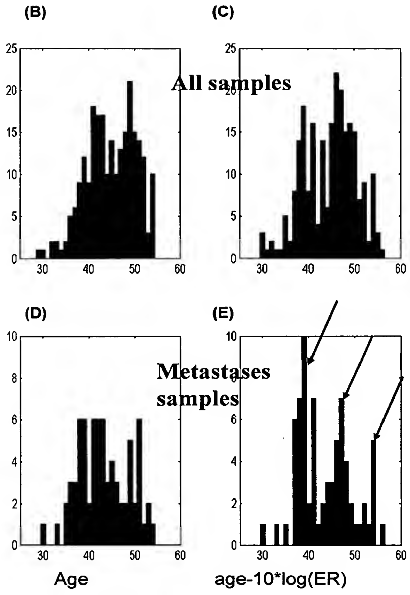
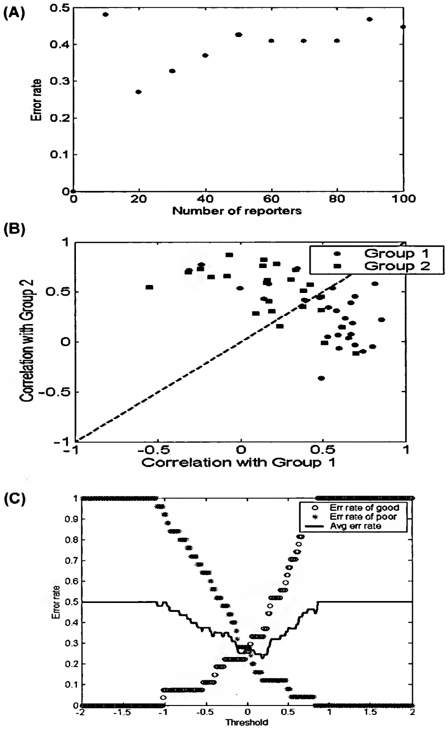



FIG. 2A




FIGS. 2B-D



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60
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(B)
Correlation with Group 2
Correlation with Group 1
Group 1
Group 2
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0.5
0
-0.5
-1
-1
-0.5
0
0.5
1
(C)
Error rate
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Err rate of good
Err rate of poor
Avg err rate
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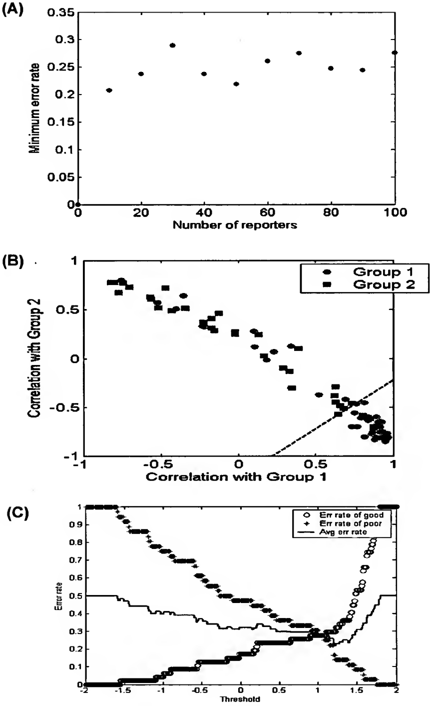
2


FIGS. 3A-C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Err rate of good
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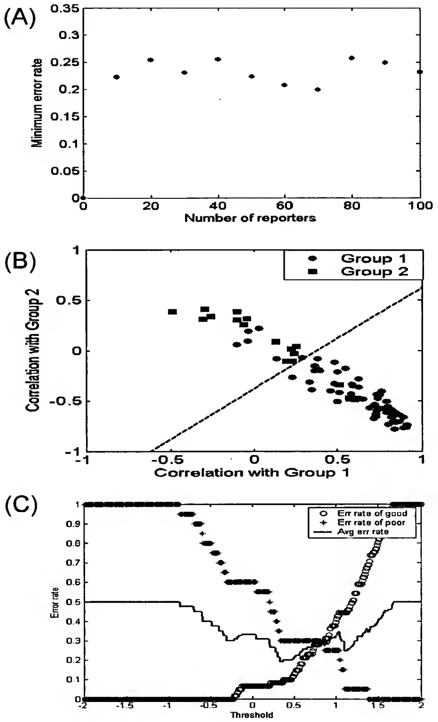
Avg err rate
Error rate
Threshold

FIGS. 4A-C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(C)
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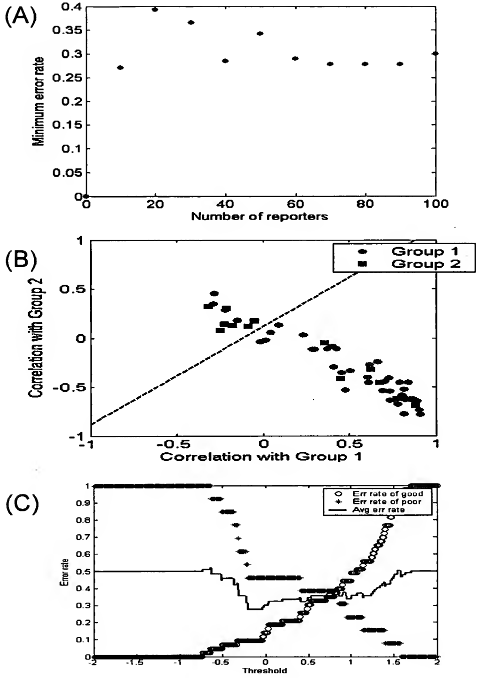
2

FIGS. 5A-C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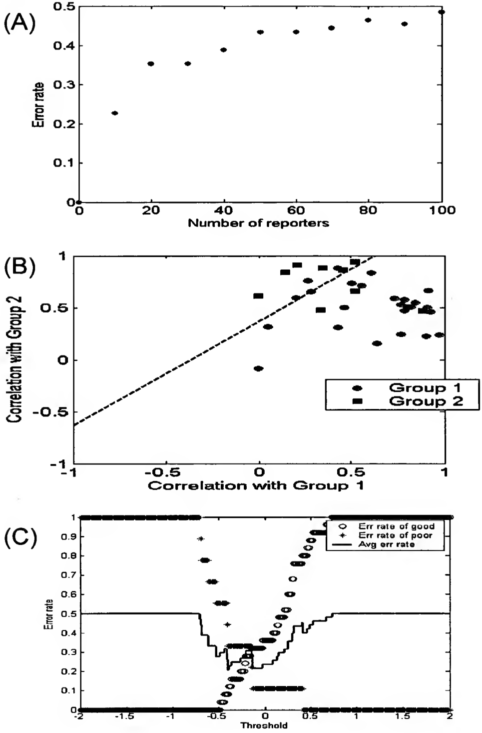
Avg err rate
Error rate
Threshold

FIGS. 6A-C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1
Correlation with Group 1
Group 1
Group 2
(C)
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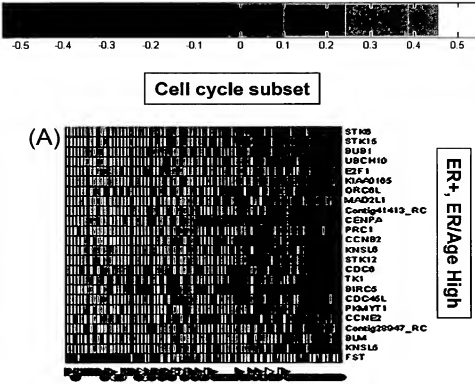
Err rate of good
Err rate of poor
Avg err rate

FIGS. 7A-C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CDC45L
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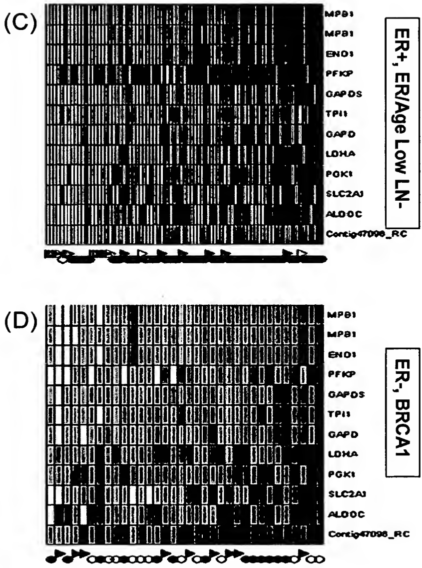
KNSL5
FST
ER+, ER/Age High
(B)
ER-, sporadic

FIGS. 8A, 8B

Glycolysis subset




FIGS. 8C, 8D

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      Lamb, John
      Stoughton, Roland
      Friend, Stephen
      Yudong He

<120> Classification of Breast Cancer Patients Using a Combination
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<212> DNA
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<213> Homo sapiens

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<213> Homo sapiens

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